STRESS, DIET AND CANCER

A study on incidence and growth of malignant tumors in rats

cover illustration: Kees de Vries

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A study on incidence and growth of malignant tumors in rats

STRESS, VOEDING EN KANKER

Een onderzoek naar de incidentie en groei van maligne tumoren bij ratten

PROEFSCHRIFT

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To the memory of
Dick Westbroek

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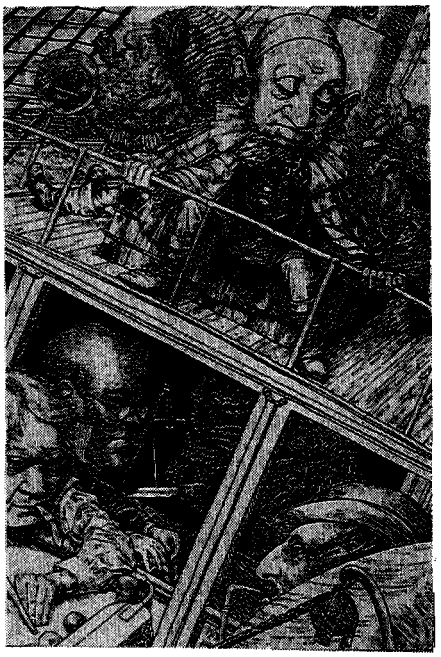

The crew was complete: it included a Boots-
A maker of Bonnets and Hoods-
A Barrister, brought to arrange their disputes-
And a Broker, to value their goods.

From "The Hunting of the snark", by Lewis Carroll

1. GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

The first example of a well documented epidemiological study that demonstrated a relationship between environmental conditions and cancer was the study of Doll and Hill [40], who found a ten-fold increase in the number of deaths attributable to cancer, in relation to the use of tobacco. First in the U.S., and later in other countries as well, this discovery led to the recommendation to avoid the habit of smoking tobacco [163].
After these first epidemiologic studies, an avalanche of articles appeared, in which many environmental factors were mentioned that might increase tumor risk [4,36,41,44,65,121,129,152,172,173]. Of the numerous factors mentioned, dietary factors, occupational factors, and stress were the most prominent.
Higginson [65] stated that environmental factors may contribute to 90% of total tumor incidence, implicating that a tremendous preventive potential might be achieved by changing personal life styles.
Although experimental animals are not always adequate substitutes for men, a basic principle in cancer research should be that suggestive evidence from retrospective epidemiological studies has to be confirmed in animal studies before a "suspected" carcinogenic factor is declared "guilty" [66]. That one will encounter problems when a potentially carcinogenic factor is investigated in animal studies is no reason to avoid such experiments. To facilitate extrapolation to the human situation one has to design the experiments very carefully. For instance, the carcinogenic regimen (how much, how often?) and the choice of the tumor model in which the carcinogenic factor is studied should be very well considered [66].

1.1. **The Frame of the Study**

In the present study, it was our aim to study some potentially carcinogenic factors like stress, and dietary lipids within the following framework:
-the carcinogenic factors studied have to be administered in such a way that the animals are kept in a condition of "wellbeing" as much as possible. Particularly, acute injurious effects have to be prevented.
-the factors have to be present chronically (lifelong), or at least subchronically (period of at least one month).
-the tumor model has to be comparable with a human tumor, in histo-pathological appearance, as well as in biological behavior.

-the "control" of the suspected carcinogenic factor shall be in such a way that the above mentioned considerations with regard to noxiousness, etc, are identical for the control non-carcinogenic factor.
-the biological variability of the tumor models as well as the methodological variability of the parameters have to be small.

Furthermore, it is our assumption that when a certain environmental factor causes an altered tumor risk, this factor will trigger some biochemical processes, which will result in measurable changes in the homeostasis of the individual. It will be clear that a choice had to be made, which of the many biochemical processes should be monitored. With our choice we do not suggest that, the biochemical processes not studied, do not play a role in the relationship between environmental factors and increased cancer risk.

1.2. **The Hypothetical Pathways**

1.2.1. the immuno-endocrine pathway

How environmental factors might elicit their effect on carcinogenesis remains unclear. Alterations in hormone levels [14,55,61,83], immunological inadequacies [133,169], infectious diseases [41,155], and geophysical factors [85,142], were mentioned. To restrict ourselves in our quest for those mechanisms that might be responsible for the observed cancer risk from environmental factors, we have chosen the "immuno-endocrine pathway" as a main track for our studies. We prefer to use this terminology because variations in hormone levels and immune capacity are very closely related [169]. For instance, when adrenocortical hormone secretion is increased, murine mammary tumor growth is decreased [169]. As corticosteroids are also very potent immunosuppressive agents, in such situations as the given example, it is impossible to differentiate between hormonal and immunological alterations.

To summarise our hypothesis; we assume that certain environmental factors may lead to a decreased capability of the individual to recognise and/or to eliminate malignant cells, which are caused by alterations in cell-cell interactions. We may illustrate this with an example: as a result of stress, plasma corticosteroid levels increase, the high corticosteroid levels lead to a depressed cellular immune response, with subsequently a depressed

cellular immune system, resulting in an altered pattern of tumor growth and incidence.

1.2.2. the prostaglandin pathway [17,90,91,153]

As it has been shown that the quantity of polyunsaturated fatty acids (PUFA; in particular, linoleic acid) in the diet is important to prostaglandin synthesis capacity [49,125], some investigators postulated that the increased growth of mammary tumors in rats on high PUFA diets might be caused by an increased prostaglandin synthesis capacity [68,80].
Some evidence for the potential role of prostaglandins in relation to diet and cancer has been obtained so far:
-dietary factors could induce changes in prostaglandin (PG)-synthesis [49,125]
-increased tumorigenesis in rats, fed high fat diets, could be abrogated when in these animals PG-synthesis was blocked by indomethacin therapy [24]
-tumor bearing individuals showed increased plasma PG values [1,94,124,162]
-PG-synthesis by malignant tumor cells in vitro was different from PG-synthesis by cells from normal or hyperplastic tissue [10,12,88,92]
-PG's could play an important role in certain immune processes [59]
-PG's and Thromboxane-A_2 (TXA_2) played an important role in hemostasis, which seemed, on its turn, to play an important role in tumor spread [71]
-in some cases tumor associated complications, such as diarrhoea [6], or bone decalcification [136], could be decreased with therapy that blocks PG-synthesis.
-tumor cells were found to be able to synthesize "pro-coagulant factors" (TXA_2?) which stimulate a platelet aggregation [50,51,57].

In summary; the hypothesis that prostaglandins are involved in carcinogenesis is based on the finding that changes in arachidonic acid availability (e.g. induced by changes in the dietary fatty acid composition) lead to changes in prostaglandin synthesis; which changes may then be responsible for an altered immune response, which links this hypothesis with the "immuno-endocrine pathway".
N.B. although 5-lipoxygenase products may also play a role in tumorigenesis, in the context of this thesis the discussion of this pathway has been intentionally omitted.

As has been mentioned, the capacity for synthesis of in particular PGI_2 and TXA_2 seems to play an important role in hemostasis as well [60], therefore alterations in the synthesis of these PG's may affect metastasis in the following hypothetical way: malignant tumor cells entering the circulation (lymph, or blood), will eventually arrive in the capillary system. At this site they cause a local platelet aggregation by means of the tumor cell's capacity to synthesize aggregating factors, as a result of which the tumor cells are surrounded by platelets and are shielded from cells with phagocytic capacities, the tumor cells survive and new tumors are born [167].
For many aspects of the hypothesis, experimental evidence was found [71]. It is one of the purposes of the present study to bring in new information on this subject in order to find proof pro or contra the hypothesis that prostaglandins play an important role in tumor metastasis.

1.3. **The Choice of the Environmental Factors**

In the earlier mentioned epidemiological studies in man the strongest correlation between increased tumor risk and environmental factors was obtained for "nutrition" [128,172]. This latter could be further differentiated into a great number of different dietary factors, from which dietary fat seemed to be the constituent that correlated most significantly with certain types of cancer, in particular colon and mammary tumors [41,129]. Therefore, this specific environmental factor was studied in particular. The aim of an important part of the study was to investigate the relationship between diets with a constant fat content but "high" or "low" in the two main types of polyunsaturated fatty acids, and tumor growth or incidence.
Another environmental factor that raised special interest in relation to increased cancer risk in man was stress [36,62]. Therefore, in our rat tumor models, as a second environmental factor, stress was studied. Rats were exposed to weekly alterations in night/dark rhythm. This type of stress induces, for instance a chronic adaptation of dietary habits, and can be regarded as mild chronic stress [175].

In summary; tumor incidence and growth was studied in rats receiving mild chronic stress or diets in which the composition with regard to polyunsaturated fatty acids was altered.

2. GENERAL METHODOLOGY

2.1. **The Choice of the Tumor Models**

In general, 3 types of tumor models can be distinguished: physico-chemically induced, virally induced, and "spontaneous" tumors [132]. In syngeneic rat strains it often is possible to transplant such obtained tumors. After some passages in syngeneic rat strains one may speak of a "transplantable tumor line". Biological and histopathogical features of such transplantable tumor lines may vary. For instance, transplantable tumor lines can be further differentiated into: immunogenic vs non-immunogenic; carcinoma vs sarcoma; metastasizing vs non-metastasizing; hormone responsive vs non-hormone responsive, ones.

Within the frame of the study as was mentioned in Chapter 1.1., we considered "spontaneous tumor models" as the most proper ones for studying environmental factors and carcinogenesis. The obvious disadvantages of these models, however, are the long study period and the tremendous amount of work needed to process the histology to obtain the microscopic data. Nevertheless, this should not be taken as a too serious handicap. The second best model of tumor growth that resembles the "ideal tumor model" as was for instance formulated by Clifton [33], is a "transplantable tumor model", preferably one that originated "spontaneously". In our studies such models were used as well. The tumors used have a number of biological characteristics in common, but important differences in behavior exist as well. This gave us the opportunity to correlate differences in tumor characteristics with differences in either one of the aspects studied.

2.2. **The Parameters Chosen**

2.2.1. Tumor size was chosen as it was our experience that this parameter reflects tumor growth rather well. In our studies, tumor size was sometimes converted to "tumor growth rate", which expresses a $tg\alpha$, representing the slope of the tumor growth rate [Appendix, Publication IV].

2.2.2. Number of metastatic foci in the lungs was taken as parameter of tumor metastasis. The transplantable tumors selected are capable to metastasize from their inoculation site to the

lungs; at termination of the study the lungs were taken out, and fixated; after histologically processing, the tumor foci easily can be distinguished. In those studies where the tumor was inoculated intravenously it was the method of choice to determine "tumor take"

2.2.3. Serum corticosterone concentrations were determined in most of the Light-Dark shift experiments as a function test of the adrenal glands. It serves as a parameter to establish "stress" [159].

2.2.4. Plasma free fatty acids were determined to confirm the composition of the food with regard to its fatty acid distribution, and whether this was effectively ingested.

2.2.5. Body weight was measured as this parameter is simple and very useful to obtain a general impression of the condition of the animals. Moreover, differences in body weight themselves may have important consequences on tumor growth rates.

2.2.6. Food consumption was measured to establish whether the experimental diets were well taken. Substantial differences in food consumption cannot be accepted, as such differences may in itself be responsible for differences in tumor growth.

2.2.7. The measurement of Plasma arachidonic acid bioconversion products (PGE_2, 6-keto-$PGF_{1\alpha}$, and TXB_2) formed the backbone of that part of the study in which tumor growth was correlated with prostaglandin synthesis capacity.

2.2.8. In vitro platelet aggregation was measured in a "whole blood aggregometer". The parameter was chosen because alterations in prostaglandin synthesis, particularly of PGI_2 and TXA_2 may be reflected in the platelet function, from which aggregation capacity is an important characteristic [21].

2.2.9. Cellular immune response (concanavalin-A induced stimulation of lymphocytes, phytohemagglutinin induced stimulation of lymphocytes, popliteal lymph node assay, natural killer cell activity) were other important parameters in this study, since it was our working hypothesis that during stress as well as in the dietary studies the evoked immunological changes were in some way responsible for differences in tumor growth.

3. REVIEW OF THE EXPERIMENTAL RESULTS

3.1. **The Background of the Study**

In earlier studies it was our finding [99] that untreated allogenic kidney transplantations performed between inbred rats could show a large variation in graft survival time. This phenomenon among others initiated the present study.
In the referred transplantation experiments conditions were optimally standardized. Still approximately 10% of the kidney grafted rats could be considered as "long survivors". The mean survival time of animals with allogenic kidney transplantations without immunosuppressive treatment was 13 days, whereas the one out of ten "long surviving" allografted rat at least lived for more than 50 days.
Although experimental conditions, (i.e. degree of histo-compatibility, age, body weight, housing conditions, etc.) were controlled, post operative stress could have varied. This environmental factor was taken as a subject of a next study in which it was investigated, whether a certain amount of stress, given post operatively, could be held responsible for the observed prolongation of graft survival time [100]. The results were striking, not more than one day of restraint stress could alter the mean graft survival time significantly. Our conclusion was that, stress could induce a suppression of the immunological response.

At the same time, dietary influences on kidney graft survival were studied [100]. Our starting point for the study on the role of nutrition on kidney graft survival was slightly different from that related to the role of stress. It was hard to believe that variations in the rat's daily food uptake could have been responsible for the observed variation in kidney graft survival times. However, in a slightly different context the consumption of certain diets, in particular of diets high in unsaturated fat, were shown to have immunosuppressive potentials, which eventually led to prolongation of graft survival [113,114].
In our studies [100] we could partly confirm the results obtained by Mertin and associates [113,114]. In rats receiving the semi-synthetic diets, which were "high" in fat (35 en%), kidney graft survival times were prolonged, compared with those rats on standard chow, which is "low" in fat (16 en% fat; N.B. standard rat chow is also different in other constituents, such as crude

fiber). However, no significant differences between the 35 en% fat diets high or low in linoleic acid were seen. This latter was not in accordance with data obtained by others [19,97,113-116], where linoleic acid high diets were relatively more immunosuppressive compared to diets high in saturated fat. The results of these latter animal studies tempted some groups to "treat" kidney transplantation patients with diets rich in linoleic acid with promising results [110].
Actually, nowadays there is consensus about the role played by long chain fatty acids on the immune response: diets deficient in essential fatty acids (linoleic acid) induce a relatively high immune response. When the dietary linoleic acid concentration increases, the cellular immune response decreases [113,166]. However, whether an immunopotentiating effect can be obtained with low dietary quantities of linoleic acid, without attaining conditions that are the result of linoleic acid deficiency is still a subject of debate [76,77].

The fact that stress as well as certain dietary compositions could induce immunoinhibitory actions, expressed as prolonged kidney graft survival, may have its implications to carcinogenesis as well. However, so far only little evidence for a correlation between immunosuppression and increased tumor risk in man could be obtained [113,133,170]. From epidemiological studies in man, there is substantial evidence that environmental factors contribute to cancer occurrence [41,172,173]. And in the context of environmental factors and cancer, nutrition and stress (psychological stress), were often mentioned as factors of great significance in increasing cancer risk [41,65,122,135].

3.2. **Stress and Cancer**

With regard to stress, in particular the pioneering animal studies of Rasmussen [139], and of Solomon [156], produced evidence for a relation between stress and decreased immunocompetence. These findings led others to investigate also the role of stress induced depression of immunity and cancer [8,30,140,160]. Several comprehensive articles on this subject have been published [4,36,62,122,152].
The role of stress in carcinogenesis in man has been suspected since long. Galen living in the second century, believed that melancholic women were more likely to develop cancer than those who were more confident and vital. In modern science, the role of emotional stress and carcinogenesis risk is still being

considered seriously [36,62]. In this relation, the concept "cancer prone personality" was given for individuals with an inhibited life-style, melancholic and easily giving up, and the propensity to withdraw from unacceptable realities [4,62,122].
In experimental animals as well as in studies in men, the mechanism responsible for the increased tumor risk, was supposed to be stress induced changes in the neuroendocrine system [141,157]. For instance, in murine studies, severe acute stress resulted in significantly increased corticosteroid levels accompanied with a decreased cellular immune response [87,141,156]. In humans, such effects on the immune system as a result of emotional stress could also be established [8,36,62]. Depressed lymphocyte function [8,36,37,120], increased corticosteroid levels [36,152], and a higher susceptibility to Epstein-Barr virus infections were noted [95].
In our first animal experiments on the influence of stress on kidney graft survival [99,100], stress was induced by means of physical restraint; this stress can be considered as a form of acute severe stress [112]. In our later studies on the relation between stress and cancer, mild chronic stress was preferred as we considered such stress better comparable to the stress that humans endure as a result of their personal life style. For this purpose, light-dark (L-D) shift stress was chosen.
In a first experiment L-D shift stress was given during 35 weeks, while its capacity to induce neuroendocrine changes and alterations in the cellular immune response were tested [Appendix, Publication I]. Rats receiving L-D shift stress showed a significantly depressed cellular immune capacity in the Con A test and in the popliteal lymph node assay. However adrenal cortical activity, as measured by serum corticosterone levels, was not significantly increased. Rather unexpectedly, the weights of the adrenal glands were significantly decreased in those rats undergoing L-D shift stress. On the basis of these latter results one might conclude that L-D shifts do not cause stress, as stress is often defined as a condition accompanied with increased adrenal gland weight, and a cortical activity leading to increased levels of plasma corticosteroids [141,159]. However one has to keep in mind that the correlation between stress and increased cortical activity has always been found in studies on acute stress [141,159]. In studies on chronic stress, by others as well [8,120], such a correlation could not be demonstrated. Despite this lack of increased adrenal cortical activity, like in our study, the immune capacity was found to be decreased [8,120]. Therefore we concluded that L-D shift stress is significantly

different from acute or from repeated acute stress, but still has to be regarded as a condition with chronic alterations of the homeostasis (e.g. showing in the altered immune capacity), and as such has to be considered as "stress" (see also definition of stress as given in "glossary of terms"). Therefore, the model of L-D shift stress seems to us a proper model to study the relation between chronic stress and cancer.

With this background information the actual experiments could be planned and the influence of mild chronic stress on tumor growth and incidence was studied. The study falls in two parts, one in which the role of chronic stress on tumor incidence of spontaneous tumors in BN female rats is studied [Appendix, Publication II]; and another part, in which the role of stress on the growth of transplantable tumors is investigated [Appendix, Publication III].

3.2.1. stress and spontaneous tumor incidence
[Appendix, Publication II]

100 BN female rats underwent light-dark shift stress during their whole life span (from weaning to maximally 150 weeks). A control group received standard lighting regimen (7 a.m.-7 p.m., light; 7 p.m.-7 a.m., dark). The differences in tumor incidence between experimental and control group were not statistically significant

3.2.2. stress and transplantable tumor growth
[Appendix, Publication III]

6 weeks old BN female rats underwent L-D shift stress from 7 weeks prior to tumor inoculation until the animals were sacrificed. The rats received one of the following types of tumor inoculae:
-leiomyosarcoma
-squamous cell carcinoma
-basal cell carcinoma
-fibrosarcoma
-myeloid leukemia

The biological characteristics of these tumors with regard to hormonal responsiveness, metastatic behavior, etc., are summarised in Table I of Appendix Publication VI.
The fibrosacoma and the myeloid leukemia showed a retardation of tumor growth in those rats receiving L-D shift stress, compared

with the control rats. No significant differences in tumor growth by L-D shift stress were observed in the other tumor models. Both stress responsive tumors were distinguished from the other ones by the fact that these two tumors had proved to be "hormone responsive" (hormone responsiveness was established in parallel experiments, by determining differences in tumor growth between control rats and rats that were either adrenalectomized or ovariectomized). It seems logical to consider that this fact in particular may have contributed to the different growth characteristics of the myeloid leukemia and the fibrosarcoma.

3.3. **Dietary PUFA (n-6) and Cancer**

Pearce and Dayton [131] caused a lot of alarm with their rather careless statements based on the data of an epidemiological study entitled "Incidence of cancer in men on a diet high in polyunsaturated fat". In this study they found "evidence" ($p=0.06$) for a greater incidence of carcinomas in the group of men on diets high in unsaturated fat. Carroll et al. [25-29], studied this phenomenon in experimental animals using chemically induced tumor models. Although there was some criticism on their tumor models and experimental design, their experiments, and those carried out later by others as well [19,20,31,32,34,68, 79,80], demonstrably influenced food recommendation to men [35,164]. Diets high in polyunsaturated fat (PUFA), possibly able to prevent coronary heart disease were suspected to increase cancer risk, in particular with regard to mammary adenocarcinomas [63,121]. As mentioned earlier in this chapter, the studies, the epidemiological ones as well as the animal studies, were not always designed with the necessary care: For instance in experimental animal studies essential fatty acid deficient diets were often taken as controls (animals under such a restricted dietary regimen are actually ill [76,77]), and as soon as a small amount of linoleic acid was added to the diet the "favorable" inhibiting effect on tumor incidence, seen in rats receiving diets deficient in linoleic acid, could not be demonstrated anymore [68].
Epidemiological studies further revealed that total fat intake correlated with mammary and colon cancer risk [41,44,117,129, 172], however there was no proof that specifically the dietary PUFA content was primarily responsible. On the contrary, in particular, animal fat seemed to be correlated with a high cancer incidence on colon and breast cancer, while a vegetarian life style was correlated with a low risk in that respect [98,135].

Because we felt that additional data on this subject were needed to obtain a better fundamented opinion on PUFA and cancer risk, we designed a study in which semi-synthetic diets, containing the same amount of fat but with either 3.3 en% of linoleic acid ("low") or 17.7 en% of linoleic acid ("high"), were supplied to rats. Both diets were identical in all other constituents and contained those elements, in quantity and quality necessary for adequate development of rats [143].
As sequential bleeding (e.g. for determinations of immune response) was shown to be stressful as such [23,56], blood sampling for determinations of cellular immune response and hormone levels were carried out in a parallel study. In this study identical diets were given to groups of rats that were sacrificed for the assays after 7 weeks on diet. This implies that the data gathered in the parallel study, were from rats in which no tumors were inoculated and had not undergone earlier a possible stressful event, such as bleeding.
Con A stimulation of peripheral lymphocytes, and the popliteal lymph node assay (PLNA), which is a local graft-versus-host assay [105], were taken as a measure of cellular immune response. Furthermore, the earlier mentioned kidney transplantation experiments [100] can also be regarded as a first attempt to establish differences in immune capacity of rats fed diets either high or low in linoleic acid. These studies [100-102, Appendix, Publication IV] showed that cellular immune response was depressed in those animals receiving the 35 en% fat diets, when compared with rats receiving the standard rat chow, which was 16 en% of fat. No statistically significant differences were observed between diets high or low in linoleic acid. By which it was demonstrated that diets rich in fat could effectively alter the immune system; the quantity of fat appeared to be more important in that respect than the quality.

3.3.1. dietary PUFA (n-6) and spontaneous tumor growth [Appendix, Publication V]

Total spontaneous tumor incidence in BN female rats receiving a 35 en% fat diet "low" (3.3 en%) or "high" (17.7 en%) in linoleic acid, was not statistically significantly different. However statistically differences in the incidence of adrenocortical carcinomas and reticulo-endothelial tumors were seen: Rats receiving the diets low in linoleic acid had a higher number of these tumors. With regard to the age of the animals in which the

tumors were assessed, differences were established too; mammary gland-, and pancreatic island tumors were seen earlier in rats receiving the diets low in linoleic acid. Only multiple mammary tumors (i.e. >1 tumor in the same rat), predominantly benign mammary fibroadenomas, occurred statistically significantly more often in those rats on the diet rich in linoleic acid.

3.3.2. dietary PUFA (n-6) and transplantable tumor growth [Appendix, Publications IV and VI]

Groups of BN female rats received 35 en% fat containing diets high (17.7 en%) or low (3.3 en%) in linoleic acid at 6 weeks of age. In these animals, 7 weeks thereafter, one out of the following tumors was inoculated: A mammary adenocarcinoma, a soft tissue fibrosarcoma, a skin basal cell carcinoma, a ureter squamous cell carcinoma, an adrenal cortical carcinoma, a cervix/uterus leiomyosarcoma, or a myeloid leukemia. All these tumors were obtained from longevity studies, in which they occurred spontaneously.
Statistically significant differences in tumor growth were seen in those rats in which either the cortical carcinoma or the myeloid leukemia were inoculated. In rats given the diet low in linoleic acid growth of the cortical carcinoma was significantly increased, whereas the opposite effect was seen in rats with myeloid leukemia.
Note, that the adrenal cortical carcinoma and myeloid leukemia were the 2 tumors that showed differences in spontaneous tumor incidence as well (summarized in 3.3.1.).

3.4. **Dietary PUFA (n-3) and Cancer**

The reason for this part of the study was predominantly because data from epidemiological studies, from which certainly those of Dyerberg [46,47] should be mentioned, showed a low incidence of coronary heart disease among Greenland Eskimos, although they consumed a high fat, high protein diet. The high consumption of marine lipids by these people was considered responsible for this [7]. Fish and its predators living in cold sea waters contain many polyunsaturated fatty acids of the n-3 type (docosahexaenoic acid, DHA; and eicosapentaenoic acid, EPA), which explains also the observed tendency in Eskimos to have prolonged bleeding times [47,106], due to altered platelet function caused by a disturbed ability to synthesize certain types of PG's [53,81]

Apart from the claimed favorable effects on coronary heart disease DHA/EPA rich diets may influence tumorigenesis as well. Epidemiologically there is no evidence for such an assumption, although the low incidence of mammary tumors among Greenland Eskimo-, and Japanese women [123,173] is sometimes attributed to their dietary regimen, in which fish is an important constituent [7,126]. In experimental animal studies there is more evidence for a role of diets rich in n-3 fatty acids on carcinogenesis. Karmali, et al. [93], showed that growth of transplantable rat mammary adenocarcinomas was significantly retarded in those rats receiving an EPA/DHA mixture, as an oral supplementation to their diet, compared with rats that had the standard rat chow alone. This finding was confirmed by Carroll and Braden [29], and by Jurkowski and Cave [89]. Although others [161] could not confirm this tumor growth inhibitory action, they did find a decreased PG-synthesis by tumor tissue of rats on diets high (17% of total fatty acids) in EPA, compared with rats low in EPA (0.1 % of total fatty acids).
Our study was undertaken to shed more light on this subject. Moreover, as tumor metastasis has not been particularly well investigated, this aspect was studied separately. The studies with diets enriched with n-3 fatty acids are subdivided into 3 parts: a part in which tumor prostaglandin synthesis was studied [Appendix, Publication VII]; a part in which tumor dissemination was studied exclusively [Appendix, Publication VIII]; and a part in which a n-3 PUFA enriched diet was given at tumor inoculation. Finally we studied a possible alteration of tumor growth and metastasis by a diet given for "therapeutical" purposes [Appendix, Publication IX]. In all studies the transplantable mammary tumor model (BN472) was chosen as in this model, tumor take, growth, and spread can be studied.

3.4.1. dietary PUFA (n-3) and tumor growth [Appendix, Publication VII]

In this study the transplantable mammary adenocarcinoma BN472 was inoculated in rats that received 30 en% fat diets low or high in linoleic acid (group I and II; 3 and 10 en% of linoleic acid respectively); and diets isocaloric in fat, rich and low in fish oil (group III and IV; 7 or 2 en% of EPA/DHA, respectively). The diets were given from weaning till death of the animals, while tumors were inoculated when the animals were 9 weeks on diet. In a number of rats, plasma concentration of 6-keto $PGF_{1\alpha}$, PGE_2, TXB_2, and platelet aggregation in vitro were measured, before and

after tumor inoculation. Cellular immune response was determined exclusively before tumor inoculation.
The results showed that tumor growth was significantly inhibited in those groups of rats receiving the diets containing fish oil. There was no statistically significant difference in tumor growth between rats receiving the diets high (7%) or low (2%) in EPA/DHA. Plasma PGE_2 and TXB_2 values were significantly decreased in rats fed diets containing fish oil; no differences between the high and low n-3 diets were observed. As such the observed differences in tumor growth correlated with inhibited PG synthesis and with the presence of n-3 fatty acids in the diet (but not with the concentration of dietary n-3 fatty acids).
Cellular immune response capacity in all dietary groups was the same, therefore the observed differences in mammary tumor growth could not be ascribed to differences in the cellular immune response.
Tumor metastasis, which could be established in this tumor model as well, did not show any significant difference in either one of the experimental groups.

3.4.2. dietary PUFA (n-3) and tumor dissemination [Appendix, Publication VIII]

The same experimental design was chosen as was described under 3.4.1. However in stead of inoculation of a solid piece of mammary tumor in the flank, intravenous inoculation of in vitro cultured tumor cells of the same tumor line (BN472, mammary adenocarcinoma) was carried out. To determine tumor cell PG synthesis-capacity, 6-keto-$PGF_{1\alpha}$, PGE_2 and TXB_2 were measured in the culture medium of the tumor cells. Moreover the capacity of the tumor cell to induce platelet aggregation in vitro was tested as well.

The results of this part of the study showed that no significant differences in the number of metastatic foci in the lungs, which was taken as a measure of tumor dissemination capacity, could be shown in neither experimental group. PG measurements in the supernatant of BN472 tumor cells showed that they could produce significant quantities of TXB_2, however, this latter activity was also seen by cultured fibroblasts, which were used as a non-tumor control cell line. When the same concentration of cells was compared, synthesis of TXB_2 was even significantly higher in fibroblast culture medium. But the fibroblasts produced also large quantities of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) which is a

strong anti-aggregatory agent. PGE_2 and 6-keto-$PGF_{1\alpha}$ could not be detected in the supernatant of the tumor cell cultures. Although it is far from sure that PGI_2 and TXA_2 play a role in tumor metastasis, the results of the cell cultures indicate that TXA_2-synthesis alone cannot be regarded as a characteristic of malignant tumor cell behavior. A balance between cellular PGI_2 and TXA_2 synthesis, strongly in favor of TXA_2, could possibly be more specific for malignant tumor behavior.
Our results with tumor cell aggregation studies were in accordance with the idea that malignant tumor cells could produce "pro-coagulant" activities [57,73]. $1x10^5$ tumor cells added to whole blood in a "whole blood aggregometer", induced a strong irreversible aggregation. However, when such an experiment was carried out with cultured fibroblasts in stead of tumor cells, no such an aggregation could be demonstrated.

3.4.3. dietary PUFA (n-3) as a tumor therapy [Appendix, Publication IX]

In this experiment a slightly different experimental design was used. Only two dietary groups were studied, and in one group of rats a conversion of diets from a diet low in linoleic acid and high in saturated fat (diet group I, as used in 3.4.1. and 3.4.2.) to a diet high in fish oil (diet group III, as was used in 3.4.1. and 3.4.2.). Mammary adenocarcinomas were inoculated subcutaneously as was discussed previously (experiments 3.4.1.). The change from diet I to diet III took place at the time of tumor inoculation and was as such meant as a "therapeutical approach" to inhibit tumor growth or tumor cell spread. The rationale of this approach was that studies in men suggested that cancer growth could be inhibited and survival prolonged, when e.g. vegetarian diets were given therapeutically [151]. The theoretical background for this was that tumor metabolism might be influenced by the dietary composition possibly resulting in a reduced tumor cell turnover [151]. This hypothesis is more or less the same one as was used in our studies (see Chapter 1.2.2.).
The results of this study, however, did not show any therapeutic effect whatsoever of the fish oil diet. Exclusively, those rats that had received the diet from weaning onwards, had a significantly reduced growth of the inoculated primary tumor. Differences in the number of lung metastases between the experimental groups were small and not statistically significant.

3.5. **Prostaglandins and Cancer**

In Chapter 1.2.2., the reasons for this part of the study were explained briefly. In particular the connection between dietary PUFA and prostaglandin synthesis were mentioned. In the study, reviewed under 3.4. this relation between plasma PG levels and dietary fat is further shown. Apart from dietary modifications, a more frequently used approach to influence PG synthesis is the pharmacological approach, using drugs that can alter PG synthesis.
Clinically a small number of studies have been carried out in which by means of non steroid anti inflammatory agents (NSAIA), such aspirin or indomethacin, one attempted to inhibit tumor growth or to overcome tumor associated complications such as hypercalcemia or diarrhoea [6,136]. In studies with experimental animals more knowledge on this subject was gathered [11,13,16,54,108,109]. In vivo, as well as in vitro studies, demonstrated that tumor growth could be significant reduced by therapy with NSAIA [13,54,108,109]. However, in some in vitro studies, no effect of indomethacin on tumor cell growth could be shown [127].

Not only tumor growth, but also tumor metastasis could be reduced by therapy with NSAIA [42]. The anti platelet activity of these drugs was considered to be responsible for this. A number of pharmacological approaches were investigated, most of which were based on the theory that therapy should correct "favorable" conditions for tumor cells to metastasize (i.e. to decrease TXA_2 or increase PGI_2 levels [5,39,118,165]).
The results of these studies on the control of tumor metastasis varied [69,72,73,109,148]. One possible explanation for this variability in the results obtained was considered particularly, viz., the amount of aspirin used. For, in experimental animals the effect of aspirin on platelet aggregation is related in an inverse way to the amount of the drug given; i.e. low doses of aspirin were most effective [48,119]. Also in humans in which prolongation of the bleeding time was wanted in patients that underwent coronary bypass surgery only low doses of aspirin proved to be effective in the control of patency of the bypass [168].

In our study on the effect of platelet anti-aggregatory therapy on tumor metastasis, aspirin in a high, 200 mg/kg/day or a low

dose, 20 mg/kg/day was used. Furthermore, in another group of rats, theophyllin (a phosphodiesterase inhibitor, which acts anti-aggregatory too) 75 mg/kg/day, was given. Combinations of theophyllin with aspirin either in high or low doses were given as well.
The malignant fibrosarcoma (BN175), that metastasizes from its inoculation site to the lungs was used as a tumor model. In the discussed studies the tumor was inoculated subcutaneously.

3.5.1. tumor growth and prostaglandin synthesis
[Appendix, Publication X]

In this study tumor size of a malignant fibrosarcoma (BN175) and plasma-PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 values were determined. Moreover, platelet aggregation in vitro was measured as well. Tumor growth correlated with increased (10-fold) plasma TXB_2 and PGE_2 levels. Animals with small tumors (<20 mm in diameter) did not show any significant increase in plasma PG values. At the same time that due to tumor progression significant alterations in plasma PG values were observed, ADP-induced platelet aggregation was shown to be reduced.
With this study we confirmed that tumor growth could be correlated with increased plasma PG levels. However control (irradiated) tumor cells also showed elevated plasma PG levels. It remains therefore unclear whether the observed changes were due to tumor PG synthesis or due to a tumor initiated host response.

3.5.2. tumor growth and NSAIA treatment
[Appendix, Publication XI,103]

Like others before [9,13,109,148], we observed a reduction in tumor growth in our tumor model in those rats receiving the high dose aspirin treatment. Not only tumor growth itself was significantly inhibited, but also the number of metastatic foci in the lungs was shown to be reduced. Treatment with low dose of aspirin, theophyllin (this latter used as a drug to increase endogenous prostacyclin, by inhibiting its bioconversion [118]), or their combinations, did not show any favorable effect on tumor growth or metastatic spread, in comparison to the non treated rats.
The favorable effect seen on tumor metastasis in the groups of rats receiving therapy with high dose of aspirin might have been mediated by an inhibitory effect on the growth of primary tumor

itself, since it seems logical to presume a correlation between tumor mass and the number of metastasis. To exclude such an influence on tumor metastasis, in a next study the tumor was inoculated in the dorsum of the foot and therapy was given after tumor removal only, according to the same therapeutical regime. This experimental design should show the effect of treatment on metastasis more accurate as the previous model, as treatment was started at a time that all primary tumors were removed while being approximately the same size.
This experiment also showed a reduced number of metastatic foci in the lungs of those rats receiving 200 mg/kg aspirin [103]. All other treatment groups did not differ from the control untreated group. We therefore concluded that in order to obtain a reductive effect on tumor growth and metastasis, apparently the synthesis of more arachidonate metabolic products have to be inhibited. A more selectively blocking of TXA_2-synthesis, which may be achieved when low dose of aspirin is given, does not seem to be effective in the control of tumor growth and metastasis.

4. GENERAL DISCUSSION AND CONCLUSIONS

At cursory reading, the results of the studies on the relation between stress or dietary factors on tumorigenesis may seem to be disappointing, since light-dark shift stress as well as the dietary lipids studied apparently were not that important to tumor growth and metastasis. One might wonder if the experimental designs used indeed were proper ones, as other investigators on the subject of environmental factors and cancer were rather confident in their condemnation of stress and dietary fat as tumor promoting factors [4,28,31,80,152]. As an explanation for our contradictory results, we can advance the following arguments.

4.1. L-D Shift Stress

In those experiments by others in which in experimental animals a correlation between stress and enhancement of carcinogenesis was noticed, stress was always in the form of an acute or repeated acute stress [4,141,152]. Such forms of stress are very harmful to the individual, with acute, and long lasting (for more than 24 hours) effects on the neuro-endocrine system [22,134,141,157]. A direct result of acute stress is a depression of the immune system, which correlated with increased plasma corticosteroid levels [45,67,140,141]. Chronic stress, however, is different from acute stress, in particular with regard to the synthesis of corticosteroids [120,159]. Despite the fact that enhanced corticosteroid production in chronic stress could not be demonstrated, like in acute stress, a depression of the immune system was observed too [8,120].
L-D shift stress as used in our study proved to be a stressful procedure even after 35 weeks of L-D shifts. This could be demonstrated by significantly decreased body weights, and diminished adrenal gland weights. The results also showed that this model of chronic stress did not lead to adaptation, which is commonly observed in most types of stress that are repeatedly given [18,149]. In our studies, glucose levels were altered as well, although these alterations could also have been due to the time of blood sampling, as many hormones and other biochemical end products are subject to a circadian rhythm [58,130,175]. When the night-day pattern is changed, metabolites subject to such a rhythm will show different levels despite measuring at the same time of the day. In our experiments, to eliminate such a disturbing effect, blood samples were taken exactly a week after shifting from dark to light, when, according to other

investigators, adaptation to the new light-dark rhythm should have taken place [175]. Yet, circadian rhythms may have complicated the interpretation of hormone determinations.
L-D shift stress has its human equal; the stress that rats are subject to by L-D shifts will certainly be related to that one of human shift work [96,154,171], and therefore we considered it superior to other methods and more relevant to the human situation.
Despite the demonstrable inhibiting effect of L-D shift stress on the immune capacity, not all tumor models demonstrate a difference in tumor growth [Appendix, Publication II and III]. This is not so surprising, since 3 out of 5 tumor transplantable tumor models used in our studies, were non-immunogenic, according to the criteria as formulated by Prehn and Main [137]. The as such classified non-immunogenic tumors should consequently be insensible to induced immunological alterations.
In humans, most tumors are non-immunogenic as well [170], so it should be expected that immunosuppression only leads to enhancement of certain types of lymphoblastic tumors [133]. It might have been coincidence, but in our experiments, one of the two tumors that showed reduced tumor growth after stress induced immunosuppression, was an (immunogenic!) myeloid leukemia. However, the cortical carcinoma that was also stress-responsive although its is a non-immunogenic tumor. For this reason, it seems more likely that the hormonal alterations that were caused by chronic L-D shift stress are to be hold responsible for the observed inhibition on tumor growth by L-D shift stress. This would explain why only the adrenal cortical carcinoma, and the myeloid leukemia, which were the hormonal responsive tumor models used, showed differences in tumor growth after L-D shift stress.

4.2. **Diets Rich in Linoleic Acid**

On comparison of carcinogenesis in rats on semi-synthetic experimental diets to that in rats on commercial chow, important differences in the immune response and tumor growth could be observed. However, differences between diets, identical in fat concentration, but high or low in linoleic acid were not that significant. Despite the fact that many authors have recorded such differences [28,32,80] as mentioned in Chapter 3.3., this discrepancy might be explained by the fact that the control groups in those studies received too low quantities of linoleic acid, and have to be considered (partially) essential fatty acid deficient. Also a deficiency in dietary essential amino acids, or total protein have been shown to offer a kind of "protection" against tumor development [144,145]. Moreover most results were

obtained in animal models in which tumors were induced by administration of chemical carcinogens. Such tumors are immunogenic [138], and may better respond to the dietary induced immunosuppressive differences. But because spontaneous human tumors are seldom immunogenic [170], we preferred to use non-immunogenic tumor models. Such animal models probably give a more realistic picture of the possible effect of dietary factors, and are more relevant to the cancer risk situation in man [2,144].
Despite the fact that actually no important differences in immune response could be shown between those groups of rats either on a diet high or low in linoleic acid, differences in tumor incidence and growth were observed. At the same anatomical site tumors that proved to be "stress-responsive", were "dietary fat-responsive" as well. Incidence and growth of tumors of the reticulo-endothelial system and adrenal cortical tumors differed in the experiments on linoleic acid, high or low, both in the model of spontaneous tumor incidence and in the models of transplantable tumor growth. However, in general the diet high in linoleic acid was not tumor promoting, contrary to the diet low in linoleic acid. This is the opposite to what was found by the earlier quoted investigators [28,32,80]. The differences in tumor growth and incidence observed in our experiments do not seem to be dependent of the immune response, since only non-statistically significant differences in the immune response were observed between diets high or low in linoleic acid. As a mechanism for the relation between dietary fat and enhanced carcinogenesis, others have mentioned also: alterations in membrane fluidity (due to differences in phospholipid composition) and alterations in prostaglandin synthesis capacity [26,78,166,169]. As an explanation for the differences in tumor growth by dietary fat hormonal alterations were mentioned too [86], which is then the same explanation as was discussed earlier in this chapter for the relation between stress and tumorigenesis.
When enhancement of tumorigenesis is found after feeding high levels of dietary fat, many indications exist that hormones play an important role in this. In the first place, in longevity studies, pituitary gland tumors incidence is very high [Appendix, Publications II,V]; the presence of these tumors correlated strongly with mammary gland hyperplasia. This observation and the finding that pituitary gland tumors were diagnosed as prolactinomas, made it likely that increased prolactin synthesis might have occurred. Increased prolactin levels are sometimes mentioned to be tumor promoting [82,86]. Rats receiving diets high in saturated fat have also prolonged increased insulin levels, in comparison to rats on diets high in unsaturated fat, probably due to a longer intestinal resorption period of the

first mentioned diet [84]. So one may conclude that sufficient data is present to support the importance of a hormonal pathway. In future research on the relationship between dietary fat compositions and carcinogenesis this pathway justifies more research. Certainly, prostaglandins, being local hormones as well, should then be included in these studies.

As an important side issue of the investigations on stress or linoleic acid and cancer in the spontaneous tumor incidence model, a strong correlation could be shown between body weight at a certain age of the rat and tumors at some particular anatomical sites [Appendix, Publications II and V]. This was especially the case in animals with a relative overweight and the incidence of mammary- and Langerhans' islet tumors. In the studies of Ross et al. [147] such a correlation was found as well.

4.3. **Diets Rich in Fish Oil**

The supposition that prostaglandins could play an important role in the relation between stress or dietary linoleic acid and altered tumor risk may seem to be farfetched, still, this pathway is often proposed as an explanation of the favorable (inhibiting) effect of diets high in fish oil on cancer growth [38,106,161]. In our studies we further confirmed this hypothesis. Not only a difference in tumor growth could be demonstrated in those rats receiving the fish oil (Menhaden oil) enriched diets compared with rats fed diets high in saturated or unsaturated fat of the n-6 fatty acid, also a correlation with decreased eicosanoid synthesis could be shown, although this was not the case for PGI_2, since 6-keto-$PGF_{1\alpha}$ levels in rats receiving the fish oil rich diets did not show any alterations.

4.4. **Metastasis**

Although we considered the possibility that tumor metastasis might as well be influenced by stress [160,174], or by the quantity or quality of PUFA in the diet, this was not shown in our experiments. Many investigators could influence metastatic spread of malignant tumors by means of a therapy that altered the PGI_2/TXA_2 balance. Despite the fact that we could significantly reduce TXA_2 synthesis by means of fish oil [Appendix, Publication VII, VIII, IX], we could not find any favorable effect of this reduction of the eicosanoid synthesis on "spontaneous metastasis" (from subcutaneously implanted tumors) nor on "artificial metastasis" (from intravenously inoculated tumor cells).

Still, in our experiments, in which the tumor growth rate could significantly be reduced by NSAIA therapy, spontaneous metastasis was also significantly decreased. However, when the same therapy was given to rats in which tumor cells were inoculated intravenously, the favorable effect on metastasis disappeared. This proved that the originally acquired decreased tumor spread was not obtained by an effect on tumor take, e.g. the number of metastasis, but by an inhibition of successful tumor metastasis growth.
Despite the favorable effect on tumor metastasis that could be demonstrated by some investigators [42,43,69-75], our results suggest that treatment which interferes with platelet function, by blocking PG synthesis, plays only a minor role in the prevention of metastasis. Also other anti-aggregatory or anti-coagulant therapies using different types of drugs were not very successful in this respect [104]. Without derogating the importance of the experiments mentioned above - in particular the excellent work of Honn and others [69-75] - we think that so far the prospectives of therapies directed to obtain a "favorable" hemostatic conditions by altering the PGI2/TXA2 balance (i.e. compensate for tumor TXA_2 production or for decreased vessel PGI_2 synthesis), are so far not very promising with regard to the prevention of tumor metastasis.

4.5. **Diet Cancer Therapy**

In groups of rats a change of diets was given at tumor induction from a diet with "tumor promoting" (diet group I in Publication VII; 30 en% fat, high in saturated fatty acids) to a diet with "tumor inhibiting" capacities (diet group III in Publication VII; 30 en% fat, with unsaturated fatty acids of the n-3 type). This experiment was for an important part inspired by the hypothesis that the inhibiting effect on arachidonate metabolism of the latter diet may decrease tumor growth and metastasis as well, when supplied after tumor inoculation only.
However, for such a cancerostatic effect no evidence could be found. We therefore conclude that the favorable effect on tumor growth is primarily initiated by a retardation of the "tumor lag time", which we define as the time between inoculation of the transplantable tumor and first detectable tumor growth. It was our experience that when a tumor starts to grow, not many differences in growth can be obtained anymore. Most is reached in this first phase after tumor inoculation. If this is true it should not be surprising that not much can be gained by a "tumor inhibiting" therapy, when supplied exclusively after tumor inoculation. Also in humans, one may wonder in which phase of

life a change of environmental conditions may still merit preventive measures. It seems most unlikely, that a change of life style just before or after tumor detection will give an alteration in the patient's prospects. Therefore, on the basis of the above reasoning, it does not seem sensible to propose a certain diet as a cancerostatic therapy (apart from diets to cope with cancer cachexia) [64,150].

4.6. **Aspirin Therapy**

In the experiments in which by pharmacological means the prostaglandin system was affected, only high doses of aspirin showed an inhibiting effect on tumor growth. This suggests that the cyclooxygenase pathway had to be blocked to a major extent in order to obtain that favorable effect, since low dose of aspirin nor theophyllin showed any favorable effect. Like in later experiments [Appendix, Publication X], in particular increased plasma PGE_2 levels were associated with tumor growth, a finding that could be confirmed by many other investigators [15,52,111,158]. If so, it is not surprising that treatment with aspirin 200 mg/kg/day, which blocks all synthesis of cyclo-oxygenase products, was in our hands the only treatment that inhibited tumor growth.

4.7. **Tumor Prostaglandin Synthesis**

It is far from sure that the high PGE_2 levels sometimes accompanying tumor growth are synthesized by the tumor itself, since we demonstrated that irradiated dead tumor tissue could initially also induce elevated PGE_2 levels [Appendix, Publication X]. Because isogeneic irradiated spleen cells did not show increased plasma PGE_2 levels, we presume that a host immunological reaction, specific to tumor tissue, could also have been responsible for the increased plasma PGE_2 levels in tumor bearing rats. Other investigators found that monocytic cells, attracted by the tumor, could produce substantial amounts of PGE_2 [15,59], a process which could have been involved in our study as well. Compared with normal control values, 13 days after inoculation of viable tumor or non-viable irradiated tumor tissue, still differences in PG synthesis could be observed [Appendix, Publication X]. However, thereafter plasma PG values of rats with irradiated tumor tissue returned to basal levels, while plasma PG values of tumor bearing rats further increased. This does not imply that the increased PG activity originated from the tumor itself. After 2 weeks, an immunological reaction to the tumor inoculum in those rats with irradiated tumor tissue

could have faded away, whereas in animals with vital tumor tissue, this reaction may still continue and could actually be augmented by the increasing tumor mass.

4.8. **Conclusions**

Our main conclusion from the work presented in this thesis is that the hypothesis that both mild chronic stress and diets rich in linoleic acid may increase the incidence, growth and metastatic behavior of some, or most cancer types could not be confirmed. In fact, the opposite seems to be more true: a decreased cancer incidence correlated with the diet high in linoleic acid, and with the stress given.
Enrichment of the human diet with fish oil (rich in EPA and DHA) may have advantages; not only with regard to a possible preventive effect on coronary heart disease, but also to (mammary) tumor incidence these dietary constituents are claimed to be favorable. However one also has to take into consideration that tumors at other sites may increase. The Japanese and Greenland Eskimos have a low incidence of mammary tumors but for example the incidence of stomach tumors is high [123,126]. As it seems likely that both tumor types are influenced by dietary habits, it may be possible that what is gained at one side is lost at the other. More research related to this problem is urgently recommended.
The amount of fish that is necessary to obtain a favorable effect on tumorigenesis is hard to realize in human diet. From epidemiological studies one may get the impression that much fish of a certain kind (only a few types of fish contain useful quantities of EPA/DHA) is needed.
Although our results are not yet conclusive in that matter, it does not seem necessary to consume very high quantities of fish oil in order to obtain a reductive effect on tumor growth, since no significant differences were seen between those groups of rats receiving either 7 en% or 2 en% of EPA/DHA.

With regard to stress, more or less the same conclusion may be drawn as we did for dietary PUFA: promotion of cancer incidence by mild chronic stress does not seem likely. This may not be true for acute stress, as in animal studies as well as in retrospective epidemiological studies in men a strong correlation appeared to exist between acute stress and increased tumor risk [4,141,152]. It is yet unclear which forms of stress in human have to be considered acute stress, and which as chronic ones. Hospitalization, surgery or bereavement could be acute stress, whereas persons with certain psychological characteristics might

considered to suffer from chronic stress. Therefore the often observed negative effect of surgery on cancer patients could have been the results of acute stress, caused by anesthesia, tissue damage, blood loss, blood transfusions, etc., [107,160].
Although mild chronic stress may be relatively insignificant in respect to cancer risk, its importance to other diseases will have to be regarded carefully. After all, it seems that chronic stress does increase the susceptibility to coronary heart disease, bacterial or virus diseases [3,96,154].
Some more evidence was gathered by us with regard to the relation between tumor growth and eicosanoid-synthesis. But it will need further exploration to learn whether increased plasma PGE_2 and TXB_2 synthesis is either from tumor, or from tumor bearer origin. Treatment with aspirin-like-drugs as adjuvant to anti-cancer therapy seems promising, but more fundamental research is needed before this can be applied in cancer patients.

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6. SUMMARY

This thesis is a compilation of eleven publications on the relation between environmental factors and tumorigenesis.
Two environmental factors were chosen, i.e. stress and diet, and studied in relation to their impact on tumor growth and incidence. Epidemiological studies in man have been shown that these factors in particular, are suspected to be responsible for the increase of colon- and mammary tumor incidence, observed in Western countries. For instance a positive correlation between animal fat consumption and the occurrence of colon- and mammary tumors has been found. Stress as a tumor promoting factor is most certainly less important than nutrition, nevertheless also stress received much attention in cancer research. Investigators found that certain stressful events could decrease the "threshold of resistance" to cancer.
The form of stress studied in this thesis is considered to be a mild chronic stress (weekly alterations of the night/day pattern); diets were either high or low in the quantity of dietary polyunsaturated fatty acids.
It was our hypothesis that if tumor incidence and growth could be altered by environmental factors this might have been caused by changes in the individual's immune capacity. Therefore the cellular immune capacity was in our study an important parameter. In order not to run the risk that the results obtained are valid for only one particular tumor model, we decided to carry out our experiments with several models, "spontaneous tumor incidence" as well as with various transplantable tumors which have different biological characteristics, and which allowed studies both with regard to tumor growth and to formation of lung metastases.

The cellular immune capacity of the rats subject to the experimental diets or stress could indeed be altered. In particular in the animals receiving stress, the cellular immune capacity was significantly decreased.
However, in next experiments in which animals received a transplantable tumor no correlation between immunosuppression and promotion of tumor growth was seen. With 3 types of tumors no differences in tumor growth were seen, in 2 ones even an inverse relationship between immunosuppression and tumor growth could be demonstrated. This implies that the hypothesis that mild chronic stress or diets rich in polyunsaturated fat could show tumor promoting capacities, via an immunosuppressive effect could not be confirmed: viz., although there was a relation between stress, diet and immunosuppression, this did not result in the expected increased tumor incidence and growth.

In those transplantable tumor models in which a relation between stress or diets rich in fat, and altered tumor growth were seen, e.g. a myeloid leukemia, a fibrosarcoma and an adrenal cortex carcinoma, it appeared that hormonal sensitivity of these particular tumors may have played a more important role in the observed differences in tumor growth than the depression in cellular immune capacity.
Apart from alterations in either hormone levels or cellular immune capacity, an other explanation for changes in tumorigenesis has been discussed also. Several investigators demonstrated that diets rich in polyunsaturated fatty acids alter the capacity of cells to synthesise prostaglandins (PG's). Furthermore it has been suggested that PG's can play an important role in cancer. Therefore, the relations between the quality of dietary fat, plasma levels of some eicosanoids and tumor growth were investigated also.
Tumor development of rats on standard rat chow was monitored by measuring some plasma PG metabolites, and the effect of drugs that inhibit PG synthesis (in our study, aspirin) on tumor growth, was investigated.
Rats receiving diets with a fish oil relatively rich in polyunsaturated fatty acids of the n-3 type showed a significantly decreased mammary tumor growth in comparison with rats that had received a diet with the same en% of fat, the fat being either rich in saturated fatty acids or rich in linoleic acid. Thromboxane-B_2 (TXB_2) and PGE_2 levels in plasma of the rats on the fish oil diets (Menhaden oil) were decreased in comparison to rats on the two other diets studied, which might be an explanation for the observed tumor inhibiting activity of the fish oil rich diet.
Growth of a malignant fibrosarcoma in animals on standard rat chow was shown to be positively correlated with increased levels of plasma TXB_2 and PGE_2. However, it appeared that the increased plasma arachidonate levels were not necessarily a contribution of tumor PG synthesis capacity, as rats implanted with lethally irradiated tumor tissue also showed increased PG levels. Therefore it seems plausible that the increased plasma arachidonate levels might have been the result of some "host" reaction against the tumor, probably by the invading white blood cells attracted by the implanted tissue.
Rats receiving a daily dose of 200 mg/kg of aspirin intraperitoneally, showed a significant reduced tumor growth, which supports the above discussed results.

Another aspect in our studies that received particular attention was metastatic spread.
As some investigators presume a correlation between "procoagulant activity" of tumor cells (most probably by TXA_2 biosynthesis), and the capacity of such a tumor to metastasise, we have also selectively altered platelet TXA_2 synthesis, in order to influence tumor metastatic behavior. Not only by low dose of non steroid antiinflammatory agents, such as aspirin, the balance between vessel wall PGI_2 synthesis, and platelet TXA_2 synthesis capacity is altered (and consequently conditions will be achieved that prevent circulating tumor cells from sticking to the vascular endothelium and to develop to a new tumor), but the same can be expected by diets rich in polyunsaturated fatty acids.
However, despite conditions could be achieved that might be considered to be potentially beneficial to prevent or decrease tumor metastasis (decreased TXA_2-synthesis, prolonged bleeding time in vivo and in vitro), the number of metastatic foci in the lungs of animals bearing malignant tumors were the same in all experimental groups.

The conclusions based on our experiments were:
-eicosanoids seem to play an important role in tumor growth (based on the tumor growth reduction by aspirin).
-rats receiving diets enriched with polyunsaturated fatty acids of the n-3 type have a decreased TXA_2 and PGE_2 synthesis capacity.
-so far there is no proof for the supposition that, by a decreased hemostasis, due to induced changes in PGI_2 and TXA_2 synthesis, tumor metastatic behavior can be decreased.
-growth of the tumors studied are associated with an increase of plasma PGE_2 and TXB_2 levels, due to increased host arachidonic acid synthesis activity.
-in some but not all rat tumor models used in this series of experiments a correlation between dietary fat intake and increased tumor incidence can be shown. When such a correlation existed, the diets rich in saturated fat were more "tumor promoting" than the diets rich in unsaturated fat.
-no evidence could be obtained for the supposition that mild stress correlates with increased tumor risk, an inverse relation seems more likely.

7. SAMENVATTING

Deze studie is een compilatie van een 11-tal publicaties met als onderwerp de relatie tussen bepaalde milieufactoren en het onstaan van kanker. Milieu wordt hier opgevat als het samenspel van factoren, die door onze leefstijl en/of door onze wooonomgeving wordt bepaald, waaronder eet- en drinkgewoonten, roken, stress, etc.
In dit proefschrift wordt beschreven op welke wijze, in ratten, de invloed van twee milieufactoren: "stress, en voeding", worden onderzocht op het onstaan en de groei van tumoren. Uit epidemiologische onderzoeken was gebleken dat de toenemende incidentie van met name mamma- en colontumoren voor een belangrijk deel toegeschreven kon worden aan deze twee milieufactoren. Zo vond men een positieve correlatie tussen de consumptie van dierlijk vet en het optreden van mamma- en colon tumoren. Voor wat betreft stress, bleek bijvoorbeeld dat vrouwen onder sterke emotionele stress een verhoogd risico hadden tot het krijgen van een maligne mammatumor.
Als te onderzoeken potentieel tumorbevorderende factoren, werd in het onderhavige onderzoek gekozen voor: een milde vorm van chronische stress (een wekelijks herhaalde verschuiving van het dag-nacht ritme); en voor wat betreft voeding, voor de kwaliteit en kwantiteit van meervoudig onverzadigde vetzuren.

Het spreekt vanzelf, dat wanneer een bepaalde relatie wordt verondersteld, tussen milieufactoren en verhoogd kankerrisico, hier een bepaalde mechanistische veronderstelling aan ten tengrondslag ligt. Bij zowel stress als voedingsvetten werd verondersteld dat een verandering van de immuunrespons de reden zou kunnen zijn voor de veranderingen in tumorincidentie.

De cellulaire immuunrespons van de dieren bleek inderdaad beïnvloed te kunnen worden door voeding en stress. Met name stress gaf een zeer duidelijke verlaging te zien van de immuuncapaciteit.
In de experimenten, waarbij de ratten een tumor kregen, bleek echter in het geheel niet dat deze verlaging van de immuunrespons een tumorbevorderende rol speelde. Integendeel, juist in die ratten, waarbij een relatieve immunosuppressie, als gevolg van stress of voeding, was geïnduceerd, bleek de groei van bepaalde tumoren geremd te zijn ten opzichte van dieren, met een hogere immuunrespons. Overigens werd de tumorgroei niet in al de gebruikte tumormodellen beïnvloed door de aangetoonde verschillen in immuunrespons.

Dat betekent dus dat onze hypothese dat de gekozen vorm van stress of vetrijke voeding een tumorgroei bevorderende werking had, die veroorzaakt zou worden door een verlaging van de immuunrespons slechts ten dele bevestigd kon worden: namelijk slechts voor de relatie tussen stress, voeding en immunosuppressie.
In die gevallen, dat er een relatie kon worden aangetoond tussen stress en vetrijke voeding en een verandering van de tumorgroei, zoals bij de myeloide leukemie, de fibrosarcoma en de bijnierschorstumor, leek eerder de gevoeligheid van deze tumoren voor hormonale veranderingen verantwoordelijk te zijn voor de gevonden verschillen in tumorgroei.
Behalve hormonale en immunologische veranderingen als verklaring voor een verandering in het ontstaanspatroon van kanker, worden nog enkele verklaringen gegeven. Zo toonden onderzoekers aan, dat voeding rijk aan linolzuur het vermogen van de cel om prostaglandines (PG's) te synthetiseren, kon veranderen. Bovendien stelden weer andere onderzoekers vast dat deze PG's een belangrijke rol speelden in het verloop van het ziekte van de kankerpatiënt. Daarom werd in een vervolgstudie (publicaties VII-XI), met name de relatie tussen voedingsvetten, prostaglandine-synthese en tumorgroei onderzocht.
In deze studie werd dus getracht antwoord te geven op de vragen: in hoeverre is de vetzuursamenstelling in de voeding van invloed op de synthese van arachidonzuur-metabolieten, en in hoeverre speelt dit een rol in tumorgroei? In een later experiment werd bovendien de vraag opgeworpen of tumorgroei gepaard gaat met verhoogde spiegels van PG's in het plasma, en of tumorgroei kon worden beïnvloed door therapie met aspirine, een PG-synthese remmer.

Voeding verrijkt met een visolie, relatief rijk aan onverzadigde vetzuren van het n-3 type, remde significant de tumorgroei in ratten, die een transplantabel mamma adenocarcinoom hadden gekregen, t.o.v. de dieren, die een voeding hadden, dat slechts bestond uit voedingsvetten van het n-6 type (zoals linolzuur). Bovendien kon worden aangetoond, dat thromboxaan-A_2 en PGE_2 synthese was gereduceerd in dieren op het visolie (Menhaden oil) dieet.
Tumorgroei van een maligne fibrosarcoom in dieren op standaard rattekorrels bleek gepaard te gaan met verhoging van thromboxaan-B_2 en PGE_2 plasmaspiegels. Het lijkt echter onwaarschijnlijk, zoals veelal wordt gedacht, dat de synthese-capaciteit van de tumor hiervoor verantwoordelijk gesteld kan worden, want toen letaal bestraald tumorweefsel in plaats van vitaal tumorweeefsel werd geïnoculeerd, werden ook sterk verhoogde plasma PG waardes

gevonden. Het lijkt dus meer waarschijnlijk dat de drager van de tumor, en niet de tumor zelf, verantwoordelijk is voor de gevonden verhoogde PG waardes.
De vraag of aspirine tumorgroei kon beïnvloeden kon met "ja" worden beantwoord. Wanneer ratten dagelijks een intraperitoneale injectie van 200 mg/kg aspirine ontvingen, was de tumorgroei significant geremd.

Een ander aspect, dat in onze studies extra aandacht heeft gekregen, was de mogelijkheid te onderzoeken of het aantal metastases van een tumor kon worden beïnvloed, door het remmen van bepaalde arachidonzuur metabolieten.
Er wordt door een aantal onderzoekers verondersteld, dat er een correlatie zou bestaan tussen de aggregatoire activiteit van een tumorcel (TXA_2-synthese?) en de capaciteit van zo'n tumorcel om te metastaseren. Wanneer dit zo zou zijn, dan kan door manipulatie van de prostacycline/thromboxaan balans (prostacyline van epitheelcellen van de bloedvatwand, TXA_2 van thrombocyten en tumorcellen), omstandigheden worden gecreëerd, die voorkomen dat circulerende tumorcellen aan het vaatendotheel blijven plakken en zich verder kunnen ontwikkelen tot een tumor.
Dit werd enerzijds gedaan door de eerder genoemde visolie-rijke voeding en anderzijds door behandeling met aspirine. Van beiden is namelijk bekend dat zij de bloedingstijd in vitro en in vivo, via remming van de arachidonzuur-synthese, kunnen verlengen.

Hoewel in onze studies kon worden aangetoond, dat potentieel gunstige omstandigheden aanwezig waren om het aantal metastases te verminderen (een verminderde synthese van thromboxaan-A_2, verlengde bloedingstijd in vitro en in vivo), bleek dit geen effect te hebben op het aantal metastases, dat in de longen werd waargenomen.

De eindconclusies gebaseerd op het onderzoek in dit proefschrift zijn:
-prostaglandines lijken een belangrijke rol te spelen bij groei van tumoren (vooral gebaseerd op de tumorgroei remmende eigenschap van aspirine).
-ratten, die een voeding krijgen, rijk aan omega-3 vetzuren, hebben een verminderde capaciteit van TXA2 en PGE2 synthese.
-vooralsnog is er geen aanwijzing dat door een verminderde plaatjesaggregatie, geïnduceerd door veranderingen in de synthese van PGI_2 en TXA_2, tumor metastase gedrag kan worden beïnvloed.

-tumorgroei van bepaalde rattetumoren gaat gepaard met verhoging van plasma PGE2 en TXB2 spiegels, die wordt veroorzaakt door een verhoging van synthese van arachidonzuur metabolieten door de tumor drager.
-in enkele van de gehanteerde tumormodellen kan een correlatie worden aangetoond tussen vetrijke voeding en een verhoogde tumor incidentie. Wanneer dit het geval is, is, op een enkele uitzondering na, de voeding rijk aan verzadigd vet meer tumor bevorderend dan de voeding rijk aan onverzadigd vet.
-geen bewijs kan worden geleverd voor de veronderstelling dat milde chronische stress tumorgroei en incidentie verhoogt, eerder het tegenovergestelde lijkt het geval te zijn.

8. CURRICULUM VITAE

Wil Kort werd geboren 12 september 1943 te 's-Gravenhage. Hij volgde het middelbaar onderwijs aan de Zuiderpark HBS te 's-Gravenhage en voltooide dat in 1960 aan de Willem Ruys MULO in dezelfde stad (diploma MULO-B). Tot aan zijn militaire dienstplicht werkte hij in het electro-technisch bedrijf van zijn vader. De militaire dienstplicht (mei 1963 - september 1964) vervulde hij als korporaal schrijver in de legerplaats de Harskamp. Hierna werkte hij bij de Gist & Spiritus fabriek te Delft, waar hij tegelijkertijd in de avonduren de opleiding van leerling analist en later van analist (Zoölogie) volgde. In 1967 haalde hij hiervoor het diploma. Hij was op de Gistfabriek werkzaam op het Farmacologische Laboratorium (Hoofd Dr. J. Wieriks), waar hij betrokken was bij het screenen van nieuwe farmaca in diermodellen. In november 1967, startte hij zijn werk aan de Erasmus Universiteit (toen nog Medische Faculteit Rotterdam). Hij begon op de afdeling Pathologische Anatomie II (hoofd Prof. Dr. M.J. de Vries), waar hij o.a. betrokken was bij de promotieonderzoeken van Dr. R.W. de Bruin (The effect of immunosuppression on function of kidney allografts in the rat, 1970), Dr. V. Vuzevski (Ultra structural basis of acute renal allograft rejection, 1976), en Dr. E.D. Wolff (Liver transplantation in the rat, 1976). De voor deze onderzoekslijn benodigde microchirugische handvaardigheid paste in feite beter bij een meer chirurgische georiënteerde afdeling, waardoor hij solliciteerde bij het Laboratorium voor Chirurgie (Hoofd Prof Dr. D.L. Westbroek). Hij is van 1972 tot heden aldaar werkzaam, eerst als laboratorium assistent en vanaf mei 1979 als wetenschappelijk medewerker. Zijn kennis van, en belangstelling voor de microchirurgie is de basis geweest voor meerdere publicaties op dit gebied van hem en anderen. Sinds oktober 1981 is hij actief als cursusleider van cursussen microchirugie, in het kader van het post academisch onderwijs geneeskunde. Zijn research activiteiten zijn na de zeventiger jaren geleidelijk omgebogen van het transplantatie immunologisch-, naar het kanker immunologisch onderzoek, en met name de rol die prostaglandines daarin spelen. De publicaties opgenomen in dit proefschrift getuigen van deze laatste belangstelling.

9. ACKNOWLEDGEMENTS

The study is a compilation of a good seven years of work in the Laboratory for Experimental Surgery (Head: Prof. Dr. D.L. Westbroek), Erasmus University, Rotterdam. Without the help of "my boss", Dick Westbroek, to whom I am greatly indebted, this thesis would not have been possible. If he had only lived to see my promotion; he would have been so proud of me.
I also owe much gratitude to Dr. W.J. Deetman, Minister of Education and Science, of the Dutch Government, who made it possible for H.B.O. qualified to write a PhD thesis. Many thanks also to my "centipede", Ineke Hekking-Weijma, who was of inestimable value for the quality of my research.
My co-authors Amelie Bijma, Prof. Dr. Iwan Bonta (Dept. of Pharmacology), Anneke Cobussen-Mathijsen, Lorette Hulsman, Prof. Dr. Wim Hülsmann (Dept. of Biochemistry I), Pim van Schalkwijk, Toos Stehman, Prof. Dr. Toine Vergroesen (Dept. of Biochemistry I), Drs. Pieter Zondervan (Dept. of Path. An. I), and Freek Zijlstra (Dept. of Pharmacology) [in alphabetical order], also played an essential role in the accomplishment of the study.
Most of the "crew" of the Laboratory for Experimental Surgery, biotechnicians, animal care takers and other staff members must have worked in the past 7 years, from time to time for my experiments, for which I am very grateful.
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Cor van Dijk of the audiovisual service of the Erasmus University took excellent care for the graphical presentation of most articles.
Last but not least, I want to thank the rats...

10. GLOSSERY OF TERMS AND LIST OF ABBREVIATIONS

ADP = adenosime diphosphate
AA = arachidonic acid = C20:4 n-6 = a polyunsaturated fatty acid, precursor of prostaglandins of the 2-types
BN = Brown Norway (inbred rat strain)
BN175 = transplantable rat tumor, orginated spontaneously in a BN rat; histopatological features of a malignant fibrosarcoma
BN472 = transplantable rat tumor, orginated spontaneously in a BN rat; histopatological features of a malignant mammary adenocarcinoma
cancer = any carcinoma or sarcoma or other malignant neoplasm
carcinogenesis = development of a cancer (in which we want to include "**cancer growth**" = the process between cancer initiation and reaching a certain cancer mass). Note also that, sofar, the **process** of generation of a malignant cancer cannot be distinguished from the **process** of a benign tumor; as such, in this study the word "carcinogenesis" has been used for the generation of a benign tumor as well.
circadian = relating to biologic variations or rhythms with a cycle of about 24 hours.
Con A stimulation = Concanavalin stimulation to lymphocytes
DHA = C20:6 n-3 = docosahexaenoic acid
EFA = essential fatty acid(s)
en% = percentage of digestible energy consumed
EPA = C20:5 n-3 = eicosapentaenoic acid, precursor of prostaglandins of the 3-types.
L-D shift = light-dark shift = a change in night-day rhythm, induced by alternating the 12 h light/12 h dark cycle for half a cycle
linoleic acid = C18:2 n-6 = essential (omega-6) polyunsaturated fatty acid (octadecadienoic fatty acid)
menhaden oil = type of fish oil, rich in (omega-3) polyunsaturated fatty acids (average of 17% eicosapentaenoic acid, 7% docosahexaenoic acid)
NK cell = natural killer cell
NSAIA = non steroidal anti-inflammatory agent(s)
PLNA = Popliteal Lymph Node Assay
PG('s) = prostaglandin(s) = synthesis products of arachidonic acid
PGE_2 = prostaglandin-E_2
6-keto-$PGF_{1\alpha}$ = 6-keto-prostaglandin-$F_{1\alpha}$, a bioconversion product of PGI_2 = prostacyclin

PHA stimulation = phytohemagglutinin stimulation of lymphocytes
PUFA = polyunsaturated fatty acids
RIA = radioimmunoassay(s)
stress = the reactions of the body to forces of a deleterious nature, infections and various abnormal states that tend to disturb its normal physiological equilibrium (homeostasis). But also a physical or psychological stimulus which when impinging upon an individual produces strain or desequilibrium.
tumor = neoplasm; an abnormal mass of tissue, that grows more rapidly than normal and continues to grow after the stimuli, which initiated the new growth cease.
tumor dissemination = scattering of tumor cells throughout the body via blood- or lymph vessels = tumor metastasis.
tumorigenesis = the development of a new growth or growths, see also underlined remark carcinogenesis.
tumor metastasis = the appearance of neoplasms in parts of the body remote from the seat of the primary tumor.
tumor spread = spread of tumor cells in other parts of the body then the primary tumor.
tumor take = successful growth of a tumor after inoculation
TXA_2 = thromboxane-A_2
TXB_2 = thromboxane-B_2

Definitions from: Stedman's Medical Dictionary, 23rd edition. The Williams & Wilkins Company, Baltimore.

Appendix: Publications I-XI

The Beaver brought paper, portfolio, pens,
  And ink in unfailing supplies:
While strange creepy creatures came out of their
    dens,
  And watched them with wondering eyes.

From "The Hunting of the snark", by Lewis Carroll

Publication I

EFFECT OF CHRONIC LIGHT-DARK SHIFT STRESS ON THE IMMUNE RESPONSE OF THE RAT

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Effect of Chronic Light-Dark Shift Stress on the Immune Response of the Rat

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KORT, W. J. AND J. M. WEIJMA. *Effect of chronic light-dark shift stress on the immune response of the rat.* PHYSIOL. BEHAV. 29(6) 1083–1087, 1982.—During a period of 35 weeks the night-day pattern of inbred Brown Norway female rats was changed weekly by alternating the light-dark (L-D) rhythm. After a period of 2 months, in a number of the animals, the cellular immune response was measured by means of Concanavalin A stimulation of peripheral blood (Con A) and a Popliteal Lymph Node Assay (PLNA). Serum corticosterone, plasma free fatty acids and peripheral leucocytes were determined as well. Seven months thereafter the remaining animals were sacrificed after which adrenal gland weight and spleen weight were established. Additionally, blood glucose and corticosterone were measured (corticosterone in vitro activity as well as the serum level). Both Con A and PLNA showed a significantly decreased immune response in the L-D shift stress group. Adrenal cortical activity measured in vitro as well as in vivo did not show any significant changes, neither at 2 months nor at 9 months. Therefore, the observed immunosuppressive effect of chronic light-dark shift stress can not be explained by an increased adrenal cortical activity. Other possible explanations for the effect of the light-dark shift stress on the immune response are discussed.

Stress Light-dark shift Immune response

IN experimental animals it is difficult to obtain situations in which chronic stress can actually be considered as effective chronic stress. In many situations in which chronic stress was the experiment's objective, adaptation or habituation took place in such a way that repetition of the induction of stress, after some time, was not experienced as a harmful procedure anymore.

Prolonged exposure of stress of a type hostile and adverse to the animal will irrevocably lead to "the General Adaptation Syndrome" (GAS) as described by Selye [25]. Less hostile ways of inducing chronic stress, such as lifelong exposure to noise, lead to habituation and partly loss of hearing as a method to overcome the harmful experience [4]. Other methods of mild chronic stress, which may potentially have some capacities as a method of actual chronic stress, such as isolation stress or prolonged sequential bleeding [5,28], will encounter logistic problems when used in large groups of animals.

In the present investigation, it was our aim to obtain a model, in which it was possible to study, in large groups of animals, mild chronic stress not leading to GAS or habituation, in order to study the evoked changes on the immune response and, in future studies, the changes on tumorigenesis.

Reversing the light-dark cycle of man and animal interferes with the individual's sleep-wakefulness pattern. The rat, a nocturnal animal, eats and drinks and exhibits most activity during night [8,31]. This means that a 12 hour L-D shift leads to reversal of the rat's behavior pattern. Hormone levels and those of other metabolites following a circadian rhythm have to be reset. Hence, rats undergoing chronic L-D shifting are exposed to changes of the homeostasis, which is repeated with every new cycle; as such, L-D shifting can be defined as a stressful procedure [28]. Because adaptation to light and dark comes about endogenously, this will not lead to habituation of the biological system. In experiments in which L-D shifts were studied, it was found that reestablishment of eating and drinking pattern took place after 7–9 days [31]. Therefore, in the present study, a 7 days interval of L-D shifts were chosen. Acute stress and immune response has been a subject to numerous studies [3, 12, 22, 23, 26]. Diseases subject to a decreased immune capacity were noted to occur in high frequency [22, 23, 26]; tumor induction was facilitated [2, 19, 21, 23] and graft survival was prolonged [14,22]. In studies in man, chronic emotional stress led to determinable immune changes [3]. It was supposed that these changes might lead to promotion of tumor induction [27]. Therefore, it appears to be very interesting if chronic L-D shifting in rats might mimic at least some of the phenomena as has been described for chronic stress in man.

In the present study, L-D shift stress was given to rats, after which cellular immune response was measured. Moreover, some determinations were carried out to elucidate the underlaying mechanism of the possible immune changes.

METHOD

Experimental Design

The experiment consisted of 2 groups of 50 animals each. One of the animals out of the L-D shift stress group died spontaneously at 9 weeks old due to broncho-pneumonia. For every determination at least 10 animals per group were used. Seven weeks after starting the experiment, out of both groups, 10 animals were randomly selected and blood sam-

ples were drawn to determine serum corticosterone, plasma Polyunsaturated Fatty Acids (PUFA), the number of peripheral blood leucocytes and Concanavalin A stimulation of peripheral blood (Con A). Furthermore a Popliteal Lymph Node Assay was carried out in (BN×WR)F1 hybrids (PLNA). Twenty-eight weeks later the experiment was terminated. Animals were killed by exsanguination, after which adrenal gland and spleen weight was established. Decapitation was carried out between 10 AM-2 PM on one single day, in such a way, that L-D shift stress rats were at the end (+7 days) of their period, in which the light-dark regimen was the same as the controls. Corticosterone was measured in a number of animals in blood serum as well as the "in vitro synthesis capacity"; lastly, determination of blood glucose was carried out. During the course of the experiment body weight was assessed at 14 day intervals.

Animals

Highly inbred female Brown Norway (BN) rats, obtained from the University Breeding Centre, were used. At 6 weeks old, the animals were randomly selected into the 2 experimental groups. The F1 hybrid animals, necessary for the PLNA were bred by mating BN male to inbred Wistar/Rotterdam (WR) females. When the offspring were 7 weeks old the males were used in the assay.

Light-Dark (L-D) Shift Stress

Every Friday the automatic timer, controlling the light of the animals undergoing L-D shift stress, was changed for 12 hours. Control animals were housed under standard lighting regimen: 7 a.m.–7 p.m. light and 7 p.m.–7 a.m. dark. Both animal rooms were adjecent and were at that time exclusively used for the animals in the present experiment. Moreover, the rooms were identical in temperature, humidity, airconditioning and basic background noise level. Handling of the animals was carried out during daytime, twice a week for cleaning and refreshing of drinking water. In the dark phase, infrared light was used to perform the necessary handling.

Corticosterone

Serum corticosterone was determined in 20 μl of serum, obtained after decapitating the animals, following a radioimmunoassay method described by de Jong and van der Molen [13]. Corticosterone in vitro, which method is a reliable index of in vivo cortical activity [30], was carried out according to the method of Saffran and Schally [24].

Plasma Polyunsaturated Fatty Acids (PUFA)

PUFA were determined in 0.4 ml plasma using a gas liquid chromatography technique. The method of extracting the free fatty acids was described by Gneushev *et al.* [9]. After extracting the free fatty acids, the solution was esterified with BF3 as was described by Morrison and Smith [18]. For this study only PUFA (being predominantly linoleic acid and arachidonic acid) were taken into further consideration.

Leucocytes

Peripheral blood leucocytes were determined in 0.02 ml of heparinized blood, gathered at decapitation, using a Sysmex Microcellcounter (Japan). Estimation of the % lymphocytes (necessary for the Con A) was done by making a smear with a drop of heparinized blood and counting 100 cells.

Glucose

Blood glucose was determined using a standard colorimetric method using a commercial kit (Dextrostix®, Ames).

Organ Weights

Immediately after decapitating, the spleen and adrenal glands were excised, kept on saline moistened filter paper, cleansed from surrounding adipose tissue and weighed to the nearest 0.1 mg.

Concanavalin A Stimulation of Peripheral Blood (Con A)

Con A was carried out using 10^5 leucocytes in whole peripheral blood. Harvesting was performed after 4 days of culturing. Sixteen hours before this, 0.8 μCi 3H-thymidine was added. Each determination was carried out in quadruplicate. Con A stimulation was expressed as counts/min/culture.

Popliteal Lymph Node Assay (PLNA)

A local graft versus host assay, measure of specific cell mediated immunity, was carried out according to the method described by Levine [15]. After local injection of parental cells into F1 hybrid recipients a localized form of graft-versus-host disease will be the result. When intradermal cells are injected in one of the foot pads this graft-versus-host reaction takes place in the popliteal node draining the inoculated foot. In this study the procedure used was as follows: 0.2 ml of whole heparinized blood was taken from the experimental animals via orbital puncture and injected into the left foot pad of non stressed (BN×WR)F1 hybrids. Seven days after injection of the blood into the test animals, the popliteal lymphnodes were excised, cleansed and weighed in tenth of mgs. Graft-versus-host reactivity was measured as mean lymphnode weight.

Statistical Evaluation

Statistical evaluation of the results was carried out using a Student t-test for 2 means to test the hypothesis that the difference between the means was negligible at a significance level of 5%.

RESULTS

The results of the present study are depicted in Tables 1 and 2. After 7 weekly shifts of the light-dark cycle, Con A stimulation was significantly depressed in the L-D shift stress group, $t(18)=5.12$, $p<0.001$. Also peripheral leucocytes were markedly decreased, $t(38)=5.84$, $p<0.001$. Because the values of Con A are not corrected for the number of leucocytes, cellular immune capacity "in toto" of the animals undergoing L-D shift stress will be even more altered. Cellular immunity as measured by PLNA fortified these latter results, in comparison with PLNA values of the controls, PLNA was decreased for 20% which difference was statistically significant, $t(18)=3.36$, $p<0.01$. L-D shift stress resulted in a 15% elevation of the plasma PUFA level, $t(18)=4.73$, $p<0.001$.

A difference in body weight could also be assessed: stressed rats were 5% less in body weight than the controls, $t(97)=3.41$, $p<0.01$.

However, serum corticosterone levels were not significantly changed, $t(18)=1.11$, $p<0.75$.

TABLE 1

DETERMINATIONS AFTER 7 WEEKLY L-D SHIFTS

		L-D shift	Controls
Serum corticosterone	Mean	65.3	60.4
μg/100 ml	SD	12.9	5.7
	n	10	10
Plasma PUFA	Mean	0.39	0.33
μmol/ml	SD	0.02	0.02
	n	10	10
Peripheral leucocytes	Mean	6.0	10.3
$\times 10^6$/ml	SD	0.9	3.2
	n	20	20
Con A stimulation	Mean	13,106	43,439
counts/min/culture	SD	9,368	16,212
	n	10	10
PLNA	Mean	29.2	37.0
weights in mg	SD	4.4	5.7
	n	10	10
Body weight	Mean	148.9	156.0
weight in g	SD	10.1	10.2
	n	49	50

TABLE 2

DETERMINATIONS AFTER 35 WEEKLY L-D SHIFTS

		L-D shift	Controls
Serum corticosterone	Mean	39.0	38.0
μg/100 ml	SD	9.8	10.6
	n	9	10
Corticosterone in vitro	Mean	17.5	19.5
μg/100 mg adrenal gland/hr	SD	4.0	5.2
	n	21	19
Blood glucose	Mean	4.9	5.5
mmol/l	SD	0.6	1.4
	n	39	40
Adrenal glands L+R	Mean	45.3	51.5
weights in mg	SD	3.8	5.3
	n	39	40
Spleen	Mean	337.6	344.5
weights in mg	SD	33.4	41.7
	n	39	40
Body weight	Mean	177.3	183.7
weight in g	SD	8.5	9.9
	n	39	40

At termination of the experiment, after 35 weekly shifts also some differences between the L-D shift stress group and the controls could be noted.

Blood glucose, adrenal gland weight and body weight were significantly decreased. Although these differences were not more than 5–15%, the findings were consistent and as such of importance: glucose $t(77)=2.53$, $p<0.05$; adrenal gland weight $t(77)=5.89$, $p<0.001$; body weight, $t(77)=3.35$, $p<0.01$.

Although these latter two parameters are in some way interrelated, when adrenal gland weight is given as a ratio of the body weight, this difference is still statistically significant.

Like the determinations carried out after 7 L-D shifts, serum corticosterone was not significantly changed after another 28 L-D shifts, $t(17)=0.23$, $p<0.20$. The "in vitro corticosterone activity" confirmed this finding. Corticosterone assayed in this manner was slightly decreased in the L-D shift stress group, $t(38)=1.39$, $p<0.85$.

Spleen weight was also somewhat decreased, $t(77)=0.71$, $p<0.55$; however, this difference was equalized when the spleen weights were related to the body weights.

Body weight from which the values after 7 and 35 shifts are given, differed throughout the whole observation period. Soon after the first L-D shifts, body weight increase in the two different groups started to deviate from each other, resulting in an establishment of the mean body weight of the L-D shift stressed rats some 5–10% lower than the controls.

DISCUSSION

So far, L-D shift stress has not yet been given as a method to induce chronic stress in animals; however, in studies in man, it has been demonstrated that disruption of the circadian rhythm may be regarded as a rather stressful procedure [1]. In this latter study, catecholamine, body temperature, alertness and mood were strongly affected and adrenaline secretion indicated a stress response of the organism.

In the present study, the effect of L-D shifting is clearly to be seen in most parameters. Body weight, a parameter which reflects indirectly the effect of stress as far as it influences the comfort of the animals [5,28], is lower throughout the whole observation period. Although this may be explained by changes in drinking and feeding pattern due to L-D shifting, even after 25 shifts there is no tendency to restore this lagging in weight gain. This reflects that permanent L-D shifting not only results in acute changes of the homeostasis, but that a more permanent disfunction of the metabolism also will be noticeable.

The immunosuppressive effect of the L-D shifting observed in this study may be another example of one of these more long lasting effects. The immunosuppressive effect after 7 weekly L-D shifts is rather extensive and may certainly result in a higher susceptibility to virus and bacterial infection, which has been also observed after acute stress [22, 23, 26]. The one animal out of the L-D shift stress group which died during the course of the experiment, may have been the result of such impaired immuno capacity. Most investigators explain the immuno inhibitory action of acute stress by its ability to stimulate adrenal cortical activity [23]. Serum corticosterone levels are elevated and in some studies, the immunosuppressive effect is abolished when prior adrenalectomy has been carried out [14].

However, in the present study the immunosuppressive effect on the cellular immune system is not attended by significantly increased levels of corticosterone. Also corticosterone levels after 35 L-D shifts were not elevated; on the contrary, the values tended to be lower than the control values, in serum as well as been measured in vitro. Moreover, adrenal gland mass was clearly diminished in the stress group. This finding gives us more confidence that it is right to conclude that corticosterone and glucose values of the L-D shift stressed rats are virtually decreased. We were a little reserved in this conclusion because corticosterone and glucose undergo a circadian rhythm [7,20], and it might have been possible that 7 days after shifting these values still were

not synchronized with control non-shift values. However, adrenal gland weights can not to such an extent as has been measured in this study, been influenced by circadian variations. Hence, in respect to a decrease of adrenal gland weight, the decreased corticosterone and glucose values make some sense.

Although these latter findings seem somewhat paradoxically with the effect of stress on such parameters, this is in accordance with other studies on chronic mild stress, in which uniformly no increase or also a decrease in adrenal activity was observed [3,28]. Also secondary manifestations, which are attended with increased serum corticosterone levels were only partly observed; a relative lymphocytopenia was manifest, however, loss of tissue mass of the spleen was not present (Table 2).

These observations make it difficult to hold the hypothesis that the observed immunosuppressive effect of chronic stress can be explained by a direct action of adrenal gland activity. Also in the study of Monjan and Collector [17], chronic stress induced modulation of the immune response was not attended by an increase of cortisol levels. Moreover, although the immunosuppressive effect of exogenous corticosterone administration is well known, it is very difficult to explain an immunosuppressive effect because of increased levels of endogenous corticosterone. Since such an increase of plasma corticosterone from stress reaches levels many times lower than plasma levels necessary to induce immunosuppressive effects by means of corticosteroid therapy. Still it remains possible, that changes in corticosterone levels (temporarily or permanently) cause interactions in biochemical systems with more direct relevance to the cellular immune response.

Interesting, for instance, is the simultaneous rise of corticosterone and plasma free fatty acids (FFA) after acute stress [10]. The rise in plasma FFA due to stress is not only controlled by adrenergic factors [10]. Therefore plasma FFA levels may reflect some other aspects of the action of stress on the organism than corticosteroid levels.

The consequence of different levels of PUFA in relation to immunosuppression has been a subject of many research studies [29]. In vitro T-cell activity is depressed when the substrate contains increasing amounts of PUFA [29]. A number of explications for this phenomenon have been given, such as: differences in membrane fluidity, alterations in cyclic AMP, decreased macrophage function or decreased synthesis of cholesterol [29]. Moreover, linoleic acid and arachidonic acid, which are the major part of plasma PUFA, are both precursors of certain types of prostaglandins. From these prostaglandins several types are known to have immunosuppressive capacities [16]. Therefore, differences in the level of plasma PUFA may have some correlation with the amount of cellular immunity. Our results support such a correlation, because PUFA levels in the L-D shift group were significantly higher than in the controls.

It will be clear that explaining the immunosuppressive effect of stress by increased PUFA amounts is still based on very little evidence and that much research is needed for further support of this statement. Stress, acute as well as chronic, has such a tremendous effect on the biological system, that certainly more pathways can be thought of, leading to a depressed immune capacity [25]. Interferon, which production is depressed as a result of stress [6], is also a potential good candidate for further exploration of the mechanism of stress induced immunosuppression.

The consequences of stress and to be more specific, its effect on the immune response are numerous. One effect, which may be advantageous in organ transplantation, is the delay in rejection of allografts which has been reported in animal studies [14,22]. Other effects, certainly detrimentally influencing the individual's condition are: a higher susceptability to diseases subject to immunological control [22, 23, 26] and enhancement of tumor induction [2, 19, 21, 23]. Also, in man, epidemiological studies suggest a correlation between environmental and occupational factors and cancer incidence [11]. Animal studies on stress and cancer development have exclusively been carried out utilising methods of acute stress [2, 21, 26]; the effects of chronic stress on tumorigenesis is not yet clear. A study, using L-D shift stress to investigate the effect of chronic stress on the growth of transplantable tumors as well as the influence on spontaneous tumor occurrence is presently under investigation (to be published).

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Publication II

LIGHT-DARK SHIFT STRESS, WITH SPECIAL REFERENCE TO SPONTANEOUS TUMOR INCIDENCE IN FEMALE BN RATS

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Light-Dark-Shift Stress, With Special Reference to Spontaneous Tumor Incidence in Female BN Rats [1,2]

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ABSTRACT—As a way to induce mild chronic stress, light-dark (L-D)-shift stress was applied to inbred BN virgin female rats during their whole life-span (group I, 100 animals); the incidences of spontaneous tumor and nontumor processes were recorded. A group of rats (group II, 100 animals) exposed to a standard lighting system served as the control group. Total tumors of 128 in group I and of 154 in group II were found in 74 and 86 animals, respectively. Neither were these differences nor was the pattern of spontaneous tumors statistically significant. Although in earlier studies L-D-shift stress had proved to be effective, especially with regard to its capacity to induce a substantial decrease in cellular immune response, apparently such alterations did not unfavorably affect longevity of BN female rats. Although as a side issue of this study, a strong predisposition for tumor incidence appeared to exist, in particular for the incidence of Langerhans' islet tumors, in fat animals at weaning. JNCI 1986; 76:439-446.

Promotional effects of environmental conditions on tumor incidence have been a subject of numerous studies (*1-3*). In a report on the contribution of the environment to cancer incidence by Wynder and Gori (*4*), they postulate that 80% of the observed cancer incidence can be considered "preventive potential." This preventive potential mainly involves defined environmental factors, such as diet, tobacco, alcohol, and occupation but also cultural and behavioral patterns, psychological factors, or stress (*3, 5*). Prospective studies by Grossarth-Maticek et al. (*6*) on the mechanism of psychosomatic factors in the process of cancerogenesis showed that traumatic life events or personality traits could lead to stress, eventually inducing neuroendocrinologic factors, and to enhanced cancer. In the last decade, studies on the influence of stress on illness or more specifically on carcinogenesis originated from this hypothesis or related hypotheses, leading to numerous comprehensive studies (*5-8*).

In epidemiologic studies of the influence of stress on tumor incidence, it is always very difficult to discriminate between stress and other factors that may cause increased tumor incidence. In animal studies in which the conditions can be controlled generally more easily than those in studies of humans, substantial evidence existed for a correlation between stress and increased tumor growth; furthermore, no decreased tumor incidence was noted (*9-11*). The mechanism that is thought to be, as a rule, responsible for the observed promotion is the decrease of the immune response caused by stress, for which there is much evidence from experimental as well as clinical studies (*12, 13*). However, although there is a strong correlation between stress and immunosuppression, there is not much proof that the temporarily or more permanently induced immunosuppression will result in an enhanced tumor incidence. Certainly, in humans, in which most tumors are nonimmunogenic, stress-linked immunosuppression causing tumors would have been surprising (*14*).

When a possible effect of stress on tumor promotion is studied, one has to consider essential differences found between acute and chronic stresses. Whereas acute stress immediately resulted in elevated levels of serum corticosterone and other endocrine functions, with prolonged exposure to stress the reverse effect was seen: Serum adrenal steroid concentrations became subnormal (*15, 16*). Although certain stressful events, such as bereavement and hospitalization, may be considered acute stress, most stressful conditions in the modern life-style are usually regarded as chronic stress. Therefore, in animal studies preferably a mild form of chronic stress should be given, for proper study comparisons to effects of human stress.

Prolonged exposure of hostile and adverse stress to the animal will lead to the General Adaptation Syndrome (GAS) as described by Selye (*17*). Less hostile inducement of chronic stress often led to habituation or compensation by other means than the above-mentioned adaptation, such as partial loss of hearing as seen in an experiment in which lifelong exposure to noise was used to induce chronic stress (*18*). Other ways to obtain mild chronic stress, such as isolation stress or prolonged sequential bleeding (*19, 20*), will encounter logistic problems when used in large groups of animals and are not very workable. In earlier studies by these authors,

ABBREVIATIONS USED: L-D-shift=light-dark-shift; MST=median survival time.

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L-D-shift stress was used on rats for 35 weeks. This stress procedure significantly decreased cellular immune response and body weight (*16*). This finding implicates that this procedure does not seem to lead to adaptation and is potentially useful in a mild chronic stress study.

Spontaneous tumor incidence of BN female animals was recorded earlier by our group (*21, 22*) and by others (*23*); these studies proved that, by establishing spontaneous tumor incidence, the influence of, for instance, dietary factors on carcinogenesis could be investigated (*22*). Expanding on this model we investigated the influence of mild chronic stress on tumor incidence. Apart from weighing the animals every 2 weeks and apart from recording the data gathered by autopsies, no other determinations were made so as not to induce potentially stressful procedures other than the given L-D-shift stress (*24*).

MATERIALS AND METHODS

Experimental design.—This experiment was part of a larger study on the effect of environmental conditions on cancer incidence, all having the same protocol and, apart from specific environmental conditions studied, having identical conditions (*21, 22*). The same protocol for the experimental design, strain of animals, and husbandry was followed as that in (*22*).

We used 2 groups of 100 animals each, equally divided into group I, L-D-shift stress, and group II, untreated controls. From weaning (3 wk) until termination of the experiment (150 wk) L-D-shift stress was used, while body weights and the conditions of the animals were recorded. Furthermore, at least twice daily the animals were carefully checked for sickness and death, without handling them. Those animals suspected to be sick were removed and housed individually; when moribund they were sacrificed. Animals with substantial tumor growth, which impeded their eating and drinking habits, were also killed.

The protocol for the sick and dead was the same as that described elsewhere (*21*). Body weight, which was determined every 2 weeks, was used as a criterium for the condition of the animals and for establishment of possible predispositions for survival or tumor incidence.

Experimental animals.—The BN female rats used (exclusively virgins) were at the time of the experiment at their 17th generation of inbreeding. Immediately after weaning (3 wk), the animals were weighed and equally divided into 2 groups: an experimental group and a control group. A total number of 70 litters, from which exactly 50% came from a second and 50% from a third litter, were necessary to complete the 2 groups. The time difference between the first and the last animal entering the study was 9 weeks. The animals were housed in Makrolon cages (41 cm long×25 cm wide×15 cm high) in groups of 5 in air-conditioned animal rooms with a controlled day-night rhythm. To prevent possible effects of the dominating animals, for instance, in food consumption or uncontrolled stress, we regrouped the animals every 2 weeks, keeping the numbers of rats at 5 per group. The location of the cage in the animal room was changed every 2 months. The animals received a commercial diet ad libitum (Hope Farms AM II, Woerden, The Netherlands).

L-D-shift stress.—Mild chronic stress was given by alternating the light-dark rhythm of the animals undergoing the L-D-shift stress. Every Friday the automatic timer controlling the light for these animals was changed half a cycle; i.e., from the standard lighting conditions (7 a.m.-7 p.m. light and 7 p.m.-7 a.m. dark) to dark during the day and to light during the night; the next week standard lighting conditions again were used. In earlier studies, this procedure had proved to be stressful, which could be established by decreased immune response, by a slightly inhibited increase in body weight, and by a decreased weight of the adrenal glands (*16*). Control animals were housed under a standard lighting regimen: 7 a.m.-7 p.m. light and 7 p.m.-7 a.m. dark. Both animal rooms were adjacent and were identical in temperature, humidity, air-conditioning, and level of background noise. Handling of the animals was limited to cleaning (twice/week), which was done during daytime. In the dark phase for the animals in group I, an infrared light was used to perform the necessary handling.

Autopsy.—Autopsies were conducted according to the protocol described in one of our previous longevity studies (*21*). Each organ system was examined, and the macroscopic findings were recorded. Thymus, adrenal, and spleen weights, as a measure of immune capacity, were determined. Routine microscopic examinations were performed on samples of liver, spleen, mesenteric lymph nodes, peripheral lymph nodes, kidneys, adrenal glands, stomach, duodenum, ileum, cecum, colon, uterus-cervix-vagina, pancreas, pituitary gland, thyroid, thymus, heart, lungs, aorta, muscle, ovaries, mammary gland, and bone marrow. Other organs were only examined when suspected of neoplasms. Tissues were fixed in 4% buffered formaldehyde and, after being processed in an Autotechnicon, were embedded in paraffin. Sections at least 5 μm thick were stained with hematoxylin and eosin. Other stains were used when required by our pathologist (P. Z.). All materials were screened at least twice by the pathologist; in numerous cases additional expertise was requested from other pathologists.[5] Cardiac ischemia was considered to be present when areas of "waviness," contraction bands, nuclear pyknosis, and deep eosinophilia of the cytoplasm were visible in the heart muscle.

Statistical evaluation.—A chi-square analysis, adapted to evaluate cumulative incidence data, according to Peto's incidental analysis (*25*), was used. In the results, the calculated values were given as *G*-values with their corresponding *P*-values. The correlations between body weight and tumor incidence were calculated with the use of the Fisher's exact probability test (*G*-values).

Because the experimental data consisted of rats found dead or killed when moribund (with animals surviving up to 150 wk) and rats sacrificed at 150 weeks, the evaluation of the differences in the ages of the animals

had to be adjusted to this terminal sacrifice. By use of the Fisher's exact probability test, the number of tumor-bearing animals (for any tumor site) killed or found dead before 150 weeks was compared with the number of animals sacrificed at 150 weeks. When no significant difference could be shown, a Mann-Whitney analysis was used to compare the differences for the tumor site by age of group I and group II animals living less than 150 weeks. Differences were statistically significant at $P<.05$.

RESULTS

From the 200 animals in the study, 2 rats in group I could not be further evaluated, due to cannibalism and autolysis; from some other animals, limited numbers of organs were missing for the same reasons. However, the Peto analysis (*25*) used in determining the differences in incidence was adapted for these missing organs. In the results given in text-figures 1 and 2 and in tables 1-6, the above-mentioned 2 animals from group I were excluded.

All the animals ($n=198$), independent of the experimental treatment, litter size, age of the mother at litter, week of birth, mortality in the litter, and parity (second or third litter), all of which might influence life-span, were analyzed for possible relation to survival and tumor incidence. However, no relation between one of these husbandry variables and survival or tumor incidence was present. However, correlations between body weight of all animals at a certain age to survival in general or to occurrence of tumors at some specific tumor sites were positively present (table 1); e.g., a strong correlation could be determined between the body weight at weaning (3 wk of age) and tumor incidence and also between body weight at weaning and the incidence of Langerhans' islet tumors. When the animals were 10 weeks of age, no such correlation between body weight and tumor incidence could be detected anymore. However, at 80 weeks the same predisposition for fat animals for eventually developing a tumor, as was detected earlier, was found again. Because, at weaning, fat and thin animals were equally divided in both experimental groups, the observed predisposition of the body weight at this age to forthcoming tumor processes could not have led to a possible misconception of the results of the tumor incidence in any of the experimental groups.

Very soon after the start of the experiment, the mean body weight of the experimental group began to diverge from that of the control group. In the first part of the experiment, the mean body weight (text-fig. 1) in group I was slightly less to that in group II; although this difference was significant ($P<.01$), most of the time the difference did not exceed 10 g. A maximum of 21 g in body weight difference was reached in the animals at 128 weeks; however, this relatively large difference in body weight between group I and group II was due to the fact that a selection of fat animals in group I died in this period.

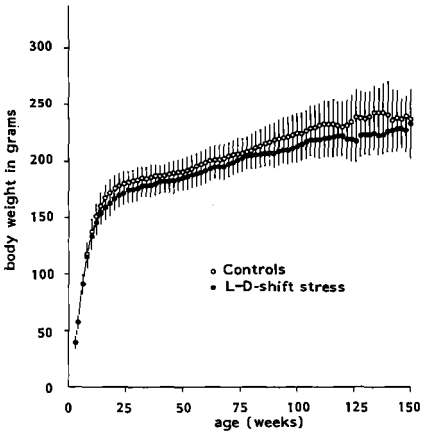
At autopsy the mean weights of thymus, spleen, and adrenal glands were not statistically significantly different. Although small differences existed in the weights of the thymus and adrenal glands (mean ± SE for thymus wt, 62.5±3.2 and 65.9±2.7, and for adrenal wt, 73.6±1.8 and 78.0±2.6, groups I and II, respectively), when these organ weights were corrected for the differences in body weight at termination they were almost equal.

TABLE 1.—*Body weight in relation to survival, occurrence of tumors (general), occurrence of mammary tumors, and Langerhans' islet tumors of groups I and II together*[a]

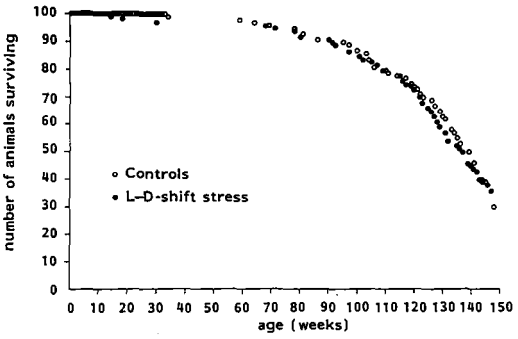
Body wt, g/rat	Wk No.	No. of rats								
		Surviving at wk:		Having tumors		Having mammary tumors		Having Langerhans' islet tumors		Total rats
		≤120	>120	Yes	No	Yes	No	Yes	No	
≤40	3	59	43	73	29	14	88	17	85	102
>40		43	53	86	10	20	76	29	76	96
		[3.37]		[10.15][b]		[1.76]		[5.08][b]		
≤136	10	53	49	79	23	18	84	21	18	102
>136		49	47	80	16	16	80	25	71	96
		[0.02]		[1.08]		[0.03]		[0.82]		
≤171	20	55	49	79	25	17	87	21	83	104
>171		45	47	80	12	17	75	25	67	92
		[0.31]		[3.85][b]		[0.15]		[1.32]		
≤185	40	51	55	85	21	16	90	24	82	106
>185		47	41	74	14	18	70	22	66	88
		[0.54]		[0.50]		[0.96]		[0.15]		
≤207	80	44	50	71	23	12	82	17	77	94
>207		48	46	84	10	20	74	29	65	94
		[0.34]		[6.21][b]		[2.41]		[4.14][b]		

[a] Group I: L-D-shift stress; group II: untreated controls. Body wt and survival parameters are divided into two categories, with the use of the median as point of division. *No. in brackets*, *G*-value (Fisher's exact probability test).

[b] Animals classified as fat at 3 wk (at weaning) and at 80 wk eventually developed significantly ($P<.05$) more often a tumor or in particularly a Langerhans' islet tumor than did the thin animals.



TEXT-FIGURE 1.—Comparison of body weight increase in groups I and II. *Vertical bars* represent the SD. Differences in body weight were statistically significant after 20 wk.



TEXT-FIGURE 2.—Comparison of the actuarial survival of groups I and II. Each group originally consisted of 100 animals; L-D-shift stress was given during the rat's whole life-span.

From all animals entering the study, 36 and 30 in groups I and II, respectively, survived the 150-week observation period and were sacrificed at that age while in good condition. From these 36 and 30 animals, respectively, 9 and 3 animals had, apart from some minor nontumor processes, no pathologic conditions. Difference in tumor incidence at 150 weeks (G-value, 2.48; $P<.10$) was probably one of the most convincing proofs in this experiment for the relative harmless effect exhibited by lifelong exposure to chronic stress.

In the actuarial survival curve (text-fig. 2) the congruent shape of both survival patterns can be seen. The MST of all animals was 138.5 and 140.5 weeks, a difference which was not statistically significant in the Mann-Whitney analysis (Z-value$=-0.05$, $P=.95$).

The animals dying in the course of the experiment from nontumor processes died mostly with signs of cardiac ischemia. In group I 4 of 12 and in group II 9 of 12 animals died from heart failure due to cardiac ischemia (the animals sacrificed at termination were not included in this comparison). All other animals, apart from the animals who some days before death had difficulties in breathing, died without any clinical symptoms or without a detectable loss of body weight, which could have predicted the oncoming death of the animal. From some of these rats, due to cannibalism, numerous organs were missing. For more details of all the important nontumor processes, *see* table 2. It has to be stressed that the pathologies given in this table were mostly not life threatening. The recorded nontumor processes were almost equally divided for both groups; no statistical significant differences in incidence or in mean age were present.

Totals of 128 and 154 tumors were found in 74 and 85 animals in groups I and II, respectively. The mean numbers of tumors per rat were 1.7 and 1.8, respectively. The pattern of the spontaneous tumor incidence is seen in table 3. In table 4 the numbers of tumors in tumor-bearing rats are given.

The differences in tumor incidence were not statistically significant for tumor-bearing rats in general or for the number of tumor-bearing rats for a particular tumor site. The fact that in group I fewer tumor-bearing rats (11) were present had, as a matter of course, also its influence on the difference in the total number of tumors found. Furthermore, it was striking that almost twice as many tumors of the cervix-uterus appeared in group II; this otherwise nonsignificant difference was caused by a high number of leiomyomas found in the

TABLE 2.—*Incidence of nontumor processes and mean ages of rats*[a]

Nontumor processes	Group I		Group II		Peto analysis (*25*); Nontumor incidence	
	No. of rats	MST, wk	No. of rats	MST, wk	G-value	P-value
Uterine infection	8	147	10	148	0.10	0.75
Hydronephrosis	52	144	49	148	0.37	0.55
Biliary cysts	35	150	49	148	1.48	0.22
Pancreatic atrophy	14	150	22	145	0.00	1.00
Mammary hyperplasia	49	144	53	148	1.26	0.27
Cardiac ischemia	14	140	22	138	1.14	0.29

[a] Group I: L-D-shift stress; group II: untreated controls.

TABLE 3.—*Incidence pattern of spontaneously occurring tumors*[a]

Anatomic site and tumor type	Group I		Group II	
	n	MST, wk	*n*	MST, wk
Pituitary gland				
Carcinoma	1	124	1	150
Adenoma	31	145	28	147
Adrenal gland				
Cortical carcinoma	9	144	9	134
Cortical adenoma	2	145	6	146
Medullary pheochromocytoma	6	147	8[b]	147
Pancreas endocrine				
Islet cell carcinoma	2	150	1	150
Islet cell adenoma	23	150	20[b]	150
Thyroid				
Medullary carcinoma	2	150	4	132
Mammary gland				
Adenocarcinoma	6	147	4	150
Adenofibroma	9	124	16[b]	118
Brain				
Meningioma	1	132	—	
Granular cell myoblastoma	3	144	3	148
Cervix-uterus				
Squamous cell carcinoma	—		1	148
Leiomyosarcoma	4	120	5	134
Leiomyoma	5	140	11	148
Liver				
Hemangioendothelioma	1	150	4	130
Gastrointestinal tract				
Leiomyosarcoma	—		1	87
Leiomyoma	—		1	137
Carcinosarcoma	—		1	150
Fibroma	1	117	—	
Papilloma	1	150	5	150
Skin and subcutaneous tissue				
Osteosarcoma	1	141	—	
Melanoma	3	124	—	
Fibrosarcoma	—		1	150
Squamous cell carcinoma	1	145	—	
Squamous cell papilloma	1	140		
Basal cell carcinoma	—		1	140
Angiosarcoma	—		1	143
Salivary gland				
Adenocarcinoma	1	137	—	
Lungs				
Adenoma	—		1	135
Kidney and bladder				
Transitional cell carcinoma	1	150	3	150
Adenoma	—		2	134
Lymphoreticular tumor				
Histiocytic sarcoma	5	150	1	79
Myelomonocytic leukemia	5	120	8	129
Lymphoblastoma	3	140	2	126

[a] Group I: L-D-shift stress; group II: untreated controls. *n* = number of animals with a tumor of the specified type. — = no tumor present.

[b] Includes No. of animals in which >1 tumor was found; *see* also table 5.

untreated control group. For the rest, the similarity in groups I and II for tumor incidences and age of tumor-bearing animals was evident. In both groups a gradual increase in the number of tumors was noted, eventually resulting in greater than 2 tumors per rat in the last 10-week period.

Table 5 shows the difference in tumor multiplicity (>1 tumor at the same anatomic site) between both groups. Whereas in group I, no tumor multiplicity was to be seen, in group II tumor multiplicity was shown seven times, at the indicated tumor sites.

In animals bearing mammary tumor, often certain endocrine tumors were found as well (table 6). Such conjunctions were seen in the same frequency in both groups of animals. The incidence of mammary tumors together with pituitary gland tumors or together with Langerhans' islet tumors was most pronounced.

The number of animals in which a tumor was found with distant metastases is given in table 7. Uniformly, in this rat strain only a limited number of tumors metastasize. In the results of this study, no exception to this finding was seen. The adrenalcortical carcinoma, the only tumor with a high metastatic rate, was found five times in group I and three times in group II, with metastases to lungs and/or liver. Also, in this respect, no significant difference between both groups was found.

DISCUSSION

In the study of the effect of chronic stress on tumor incidence or on longevity, numerous factors may influence the results even more than in the study of acute stress. In particular, the effect of uncontrolled stress due to handling and to housing conditions, such as background noise, pheromones, and infections preexisting or entering during the study, may result in unwanted variation of the results. The elimination of these unwanted factors will make an experiment on chronic stress often a cumbersome and certainly a risky venture. Although we cannot be completely sure that all factors leading to uncontrolled stress were eliminated, we are almost certain that the conditions were near optimal and, what is even more important, that background stress was identical in both experimental groups.

With regard to the chosen way to induce the stress, one may wonder if the quality and quantity of L-D-shift stress can be compared with certain types of chronic stress in humans. Although caution must be exercised when rodent stress is compared to human stress, the comparison between L-D-shift stress and the stress that, for instance, shift workers undergo seems not to be too far-fetched. Rotation shift has been a subject in several studies in which the noxious effect of this social and physiologic stress on the organism has been investigated (*26, 27*). Without much explanation, to humans the social disrupture attended by rotation shift stress is an important part of the stress whereas to rats L-D-shift stress is accomplished exclusively by means of the physiologic impairment of endocrine circadian rhythm (*28, 29*). Either as a result of this disturbance of endocrine organ function or by other mechanisms, L-D-shift stress, like most other stresses, resulted in a decreased immune capacity (*16, 27*).

In the present study, the immune capacity was not measured, because of the earlier mentioned possibility to induce unwanted stress by this procedure. However, we had every reason to believe that, like in earlier studies, which were carried out under exactly the same condi-

TABLE 4.—*Number of tumors in tumor-bearing rats*[a]

Age, wk	No. of rats dying		No. of rats with the following No. of tumors					Total No. of tumors	Mean[b]
	Total	With tumor	0	1	2	3	4		
Group I with L-D-shift stress									
0-10	0	0	—	—	—	—	—	—	—
11-20	2	0	2	—	—	—	—	—	—
21-30	0	0	—	—	—	—	—	—	—
31-40	1	0	1	—	—	—	—	—	—
41-50	0	0	—	—	—	—	—	—	—
51-60	0	0	—	—	—	—	—	—	—
61-70	0	0	—	—	—	—	—	—	—
71-80	2	0	2	—	—	—	—	—	—
81-90	2	2	—	2	—	—	—	2	1.0
91-100	5	3	2	2	1	—	—	4	1.3
101-110	7	5	2	5	—	—	—	5	1.0
111-120	6	6	—	4	2	—	—	8	1.3
121-130	15	13	2	10	2	1	—	17	1.3
131-140	12	11	1	5	3	3	—	20	1.8
141-150	46	34	12	8	17	6	3	72	2.1
Total	98[c]	74	24	36	25	10	3	128	1.7
Group II, untreated controls									
0-10	0	0	—	—	—	—	—	—	—
11-20	0	0	—	—	—	—	—	—	—
21-30	0	0	—	—	—	—	—	—	—
31-40	1	0	1	—	—	—	—	—	—
41-50	0	0	—	—	—	—	—	—	—
51-60	1	1	—	1	—	—	—	1	1.0
61-70	2	2	—	1	1	—	—	3	1.5
71-80	2	1	1	1	—	—	—	1	1.0
81-90	3	3	—	2	1	—	—	4	1.3
91-100	2	2	—	2	—	—	—	2	1.0
101-110	8	7	1	6	1	—	—	8	1.3
111-120	6	5	1	1	2	2	—	11	2.2
121-130	10	6	4	6	—	—	—	6	1.0
131-140	15	14	1	9	3	2	—	21	1.5
141-150	50	44	6	13	16	11	2	97	2.2
Total	100	85	15	42	24	15	2	154	1.8

[a] — = no tumor present.
[b] Mean = mean value calculated by dividing the No. of tumors by the No. of tumor-bearing rats.
[c] Two animals (dying at 69 and 133 wk) that could not be examined histologically were excluded.

tions, during a long period of time the immune capacity should be depressed (*16*). As others reported, for most tumor types this immunosuppression does not have to implicate that a higher incidence of neoplastic pathologies has to be observed (*30*). Inasmuch as most spontaneous tumors are not immunogenic or are very weakly

TABLE 5.—*Number of rats with >1 tumor at the same anatomic site*[a]

Anatomic site	No. of rats in group I with No. of tumors:			No. of rats in group II with No. of tumors:		
	1	2	Total	1	2	Total
Mammary glands	15	0	15	17	2	21
Langerhans' islets	25	0	25	19	2	23
Adrenal gland (cortex)	11	0	11	13	1	15
Adrenal gland (medulla)	6	0	6	6	2	10

[a] Group I: L-D-shift stress; group II: untreated controls.

TABLE 6.—*Number of rats with a mammary tumor together with a tumor at the following anatomic site*[a]

Anatomic site	Group I		Group II	
	No. of rats	Percent of mammary tumors[b]	No. of rats	Percent of mammary tumors[b]
Mammary gland + pituitary gland	6	40.0	5	23.8
Mammary gland + Langerhans' islets	4	26.7	4	19.1
Mammary gland + pancreatic gland + pituitary gland	2	13.3	3	14.3
Mammary gland + adrenal gland	1	6.7	3	14.3
Mammary gland + thyroid	1	6.7	2	9.5

[a] Group I: L-D-shift stress; group II: untreated controls.
[b] Percentage is calculated by dividing the No. of rats with the specified combination of tumors by the total No. of mammary tumor-bearing rats (group I = 15; group II = 21)×100.

TABLE 7.—*Number of tumors found with distant metastases*[a]

Original anatomic tumor site, tumor type	Total No. of tumors/ No. of tumors with metastases	
	Group I	Group II
Adrenal gland, cortical carcinoma	9/5	9/3
Adrenal gland, medullary pheochromocytoma	6/0	8/1
Pancreas, Langerhans' islet carcinoma	2/1	1/0
Bone, osteosarcoma	1/1	—

[a] Group I: L-D-shift stress; group II: untreated controls. Tumors were metastasized to lungs (from adrenal gland, cortical carcinomas; from adrenal gland, medullary pheochromocytoma), to liver (from adrenal gland, cortical carcinomas), to brains (from adrenal gland, cortical carcinomas), and to pleura (Langerhans' islet carcinoma). — = no tumor present.

immunogenic (*14*), a very limited role of stress-induced immunosuppression on the incidence of spontaneous tumors would be expected. However, other mechanisms might be involved that are apart from a pathway via immunosuppression. Certainly, when one chooses L-D-shift stress, which not only disturbs the circadian rhythm of a number of hormones, such as insulin and corticosteroids, but may decrease or increase the basal hormone levels, a direct interaction between this altered hormone function and tumor incidence may become possible (*31*). In this respect, mention has been made of the stress-increased prolactin levels on mammary tumor incidence for example (*32*). Furthermore, endogenous opioids have been mentioned (*33*).

In the results of the present study, the small but significant retardation in body weight of the animals under L-D-shift stress shows that a certain disturbance of the normal environmental conditions of the animals is present. This decrease in body weight may have had a direct influence on tumor incidence. In the results, a strong correlation between thin animals and nontumor occurrence was found. Therefore, the L-D-shift stress may have had, by the effect observed on the body weight, a positive influence on survival. It will be difficult to assess the contribution of this difference in body weight to tumor incidence. Furthermore, because the tumor incidence from Langerhans' islet tumors, which was the tumor with the most obvious correlation between body weight and incidence, was distributed equally in both groups, a relation between an L-D-shift stress-linked decrease in body weight and tumor incidence seems very unlikely. The finding that weight at a certain age had consequences on survival or tumor incidence was seen in earlier studies by our group and by others (*21, 34*). Apart from this indirectly mentioned (via a decrease in body weight) effect on tumor incidence and longevity, other factors must have been involved. An effect from L-D-shift stress more directly focused on the organism must be assumed to be responsible for the generally favorable outcome of chronic stress in regard to tumor incidence and longevity. This finding seems to receive little support from the data obtained by others; however, we must realize that most evidence for a positive correlation between stress and cancer came from studies on acute stress and cancer (*8*). In studies in which chronic or repeated acute stress was investigated, no hazardous effects were found; and in some other studies even a beneficial effect on longevity or of less chance for tumor incidence was found (*8, 35*).

Organ weights not significantly different between group I and untreated controls suggest that the chosen way of inducing stress was not effective, for stress is correlated with an increased adrenal gland weight and decreased thymus and spleen weight. With chronic stress this finding is not true: At the most no change in adrenal gland weight was seen; however, slightly decreased weights were found (*15, 16*). Furthermore, it is important to realize that all organ weights were established at termination, which means that they represent an end-point situation.

Distribution according to tumor site and tumor incidence as was found in the present experiment is not essentially different from the results that Burek (*23*) found in his study in BN female rats under conditions comparable with those for untreated controls. In this study (*23*) also a relative high incidence of cervix-uterus tumors was noted. In 19% of the animals, such a tumor was found, at an incidence in agreement with the frequency of the tumors as were detected in the untreated animals in our study (17%). Therefore, the low cervix-uterus tumor incidence in the animals in group I reveals the positive contribution of chronic stress in lessening the potential for tumor incidence at this anatomic site. So far we do not have a reasonable explanation for this phenomenon.

We conclude that chronic L-D-shift stress certainly does not contribute to increase of tumor incidence or lessen the mean age of the animals. Furthermore, the group receiving chronic stress showed favorable results in regard to the total number of tumors, the number of tumor-bearing animals, the incidence of cardiac lesions, and the incidence of tumors on the cervix-uterus. As to how far this finding may be of some comfort to those persons living under a strain comparable to the investigated L-D-shift stress is hard to assess; but for now there is not much evidence, epidemiologically as well as experimentally, that such stress is a high risk for cancer or decreased longevity.

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Publication III

THE EFFECT OF CHRONIC STRESS ON TUMOR GROWTH: AN EXPERIMENTAL STUDY IN THE RAT

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The effect of chronic stress on tumor growth: an experimental study in the rat

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To evaluate the effect of chronic stress on tumor growth, Light-Dark (L-D) shift stress was given to Brown Norway female rats prior to (for 7 weeks) and after syngeneic tumor inoculation. Tumor growth of a leiomyosarcoma, a squamous cell carcinoma, a basal cell carcinoma, a fibrosarcoma, and a myeloid leukemia was followed. The fibrosarcoma and the myeloid leukemia showed a significant retardation of tumor growth in the rats on L-D shift stress, compared with animals receiving a standard lighting regimen. It seems that a specific characteristic of these 2 tumor types, most probably their hormone responsiveness, is responsible for the observed differences in reactivity towards the type of stress studied.

There is substantial evidence, suggesting that personal lifestyle and environmental conditions contribute to tumor growth or incidence (8, 26, 27). Although, in this respect, most certainly dietary components are the most important, there are several reports showing that certain events leading to stress might as well influence tumorigenesis (6, 9, 10, 17, 21, 22). Persons exposed to overcrowding, isolation or the conditions of certain occupations, are subject to chronic stress. However, circumstances for individuals with « cancer prone personalities » suppressing their emotions, can be considered stress as well. Although there is no common opinion on the mechanism for this relation between stress and tumor enhancement, most investigators consider the observed immunosuppressive effect of certain types of stress of main importance (20, 21, 22, 24). Also in experimental animals the same phenomena were observed: certain types of stress were immunosuppressive and could lead to tumor enhancement, although inhibition of tumor growth was shown as well (22).

In the present study it was our purpose to investigate a type of chronic mild stress, with proven immunosuppressive properties (13), on tumor growth of different types of syngeneic rat tumors. The method of stress chosen was Light-Dark shift stress (L-D shift stress). L-D shift stress, which is more or less similar to stress, which shift workers are exposed to (7, 25), leads to alterations of hormonal and immune function in experimental animals as well as humans (1, 7, 13). Moreover, in animal studies, not many other ways of inducing chronic stress are available, as these mostly lead to habituation or compensation of the subject (4, 5).

In the present study L-D shift stress was investigated in a number of transplantable tumor models with different characteristics, this to enable a better judgement of possible mechanisms involved in stress induced tumor alterations.

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In a parallel study, which results have been published elsewhere, the same stress regimen was given to rats during whole life span to determine spontaneous tumor incidence (15).

Materials and methods

Experimental Design: Inbred Brown Norway (BN) or Wistar/Rij (WR) female animals, 6 weeks of age, received after randomization L-D shift stress (Group I) or standard lighting regimen (Groug II). The experimental groups, consisted of 10 animals each. Seven weeks later into each animal one particular tumor was inoculated, after which stress was continued.

Tumours: BN175, a soft tissue fibrosarcoma; WR1618, a skin basal cell carcinoma; BN569 a ureter squamous cell carcinoma; BN248 a cervix/uterus leiomyosarcoma and BN1312 a myeloid leukemia were used. BN tumors originated spontaneously in Brown Norway rats, the WR tumor after neutron irradiation in a Wag/Rij rat. Tumors BN175, BN248, BN569 and WR1618 were inoculated subcutaneously (pieces of approximately 5 mm^3) into the right flank of the animal; BN1312 was inoculated intravenously, 1 $x \cdot 10^6$ cells. Tumor characteristics are given in Table I.

Immunogenicity of the tumors was established according to the *in vivo* transplantation assay as described by Prain and Main (19). Measurement of the dimensions of primary tumor growth was established weekly, using calipers. The mean of length and width was taken as a measure of tumor size. For tumor BN175 and BN1312 a different procedure was followed. Tumor growth of tumor BN1312 was determined by weighing the spleen 16 days after inoculation of the tumor. From tumor BN175 the primary tumor was surgically removed 8 days after inoculation. 7 days later the animals were sacrificed, after which the number of metastases in the lungs was determined by counting the white nodules on the surface of the left lung.

Light-Dark (L-D) shift stress: Stress was given by weekly alterations of the night-day cycle, which was established by changing the automatic timer controlling the light of the animal room of the animals on stress for 12 hours. Control animals were housed in an animal room adjacent to the L-D shift animal room and were on standard lighting regimen: 7 a.m.-7 p.m. light and 7 p.m.-7 a.m. dark.

Husbandry conditions: Apart from the changes in night-day pattern, the animals were housed under identical conditions with regard to temperature (20-22°C), humidity (60-80%), airconditioning, and level of background noise (45-55dB). Handling of the animals was carried out during daytime, twice a week for cleaning and refreshing of drinking water. In the dark phase, infrared light was used to perform the necessary handling. The rats were fed a standard pelleted rodent diet (Hope Farms, AM II, Woerden. The Netherlands), *ad lib.* Acidified water was provided *ad lib.*, as well. As differences in food uptake, due to the stress procedure could substantially influence the results of the experiment, this was measured in a parallel study, at the time of the present study, in a number of *non tumor bearing* animals of the same inbred strain, following a procedure as described earlier (14). Mean food uptake (± S.E.) in Group I was 8.79 (± 0.30) g/rat/day; in Group II 8.75 (± 0.20) g/rat/day.

This difference was not statistically significant. Body weight gain, also measured in non tumor bearing animals between 7-13 weeks of age (the period, in which the experiment took place), was 59.1 (± 0.5) and 65.3 (± 0.5), in Groups I and II, respectively, a difference, which was statistically significant ($p < 0.001$).

Statistical evaluation: Tumor growth of BN248, BN569 and WR1618 was expressed as the mean of the individual regression coefficients b ($tg\alpha$) obtained after calculating the best fitting straight line (linear regression: $y = a + b\,x$) from the weekly determinations of the tumor size.

For tumor BN1312 the mean spleen weight was given, while for tumor BN175 the tumor size at day 7 was given. Statistical significant differences in primary tumor growth was established using a two-tailed Student t-test; the differences in metastases in the lungs of tumor BN175 bearing animals were tested using a Yates-analysis (28). The number of metastases in the lungs were scored semi-quantitatively, by dividing the counted nodules into one out of five categories: $< 11 = 1$. 11-25 = 2, 26-50 = 3, 51-100 = 4 and $> 100 = 5$. The differences were tested on a significance level of 5%.

Results

Tumor growth of BN248, BN569 and WR1618 was not affected by stress (Table II). The slopes of the tumor growth curves, given in Table II as $tg\alpha$ values are almost identical in both groups of rats. Tumor BN175 and BN1312, however, showed statistically significant inhibited tumor growth in the group of animals receiving L-D shift stress ($p < 0.05$).

By comparing the tumor characteristics as given in Table I with the results of the effect of L-D shift stress on tumor growth (Table II), it appears that the positive hormone responsiveness, which was established for tumors BN175 and BN1312, might be responsible for the observed differences between tumors not sensitive to L-D shift stress and those which are. An alteration of tumor growth of WR1618,

Table I - *Characteristics of tumor models*[a].

	BN175	BN248	BN569	BN1312	WR1618
Wks before take[b]	< 1	1	2	1	1
Growth rate[c]	4	7	14	1-2	13
Immunogenicity	—	—	—	—	+
Morphology[d]	mes	mes	epi	mes	epi
Strain	BN	BN	BN	BN	WR
Metastazing from site	+	—	—		—
Original anatomical site	soft tissue pancreas	cervix/ uterus	ureter	lympho ret. system	skin
Hormone responsive[e]	+	—	—	+	—

[a] rats on standard lighting regimen.

[b] number of weeks before detectable tumor growth.

[c] number of days between tumor size 10 mm and 20 mm diameter, except for tumor BN1312, for which was taken the number of days between 5 x 10^5 and 1 x 10^6 tumor cells in the spleen.

[d] mes = mesenchymal tumor; epi = epithelial tumor.

[e] BN175 grew more rapidly in female rats; BN1312 grew more rapidly in ovariectomized and in adrenalectomized rats. BN248, BN569 and WR1618 did not show any difference in tumor growth, either inoculated in male or in female rats.

the one immunogenic tumor, was not found, implicating that the inhibiting effect of L-D shift stress on the immune capacity seems to be less important.

With regard to tumor spread, established in rats bearing tumor BN175, the mean score (± S.E.) of the number of metastases in the lungs was 2.6 ± 0.5 and 3.6 ± 0.6, in Group I and II resp. This difference was not statistically significant ($t = 1.85$; $p = 0.06$). Involvement of the right axial lymph nodes was (pos/neg) 4/6 in Group I and 6/4 in Group II, this difference was not statistically significant as well.

Discussion

Although the immune capacity from the animals used in the present study was not determined, there was no reason to presume that in the L-D shift stressed animals, cellular immune response and hormonal imbalance would be altered differently as was seen earlier. In spite of this decrease in cellular immune response and changes in hormone function (decreased adrenal cortical hormone activity), for most tumor types no differences in growth could be shown. Assessable changes in tumor growth were only determined in the fibrosarcoma and in leukemia. As these tumors both were hormone responsive, it seems logical to suppose that the hormonal alterations accompanied by L-D shift stress were responsible for the observed inhibition of tumor growth. An observation, which was also established in humans and in other experimental tumor models (11).

Apart from changes in immune ca-

Table II

Tumor	Group I L-D shift stress Mean	(SE)	Group II Controls Mean	(SE)	Group I vs Group II T-value	(f)	p-value
BN248	10.80	(0.73)	11.45	(0.56)	—0.69	(15)	0.50
BN569	4.48	(0.25)	4.83	(0.57)	—0.98	(13)	0.34
WR1618	5.32	(0.52)	5.63	(0.52)	—1.03	(13)	0.32
BN175	17.7	(1.4)	21.4	(1.1)	—2.27	(18)	0.04 *
BN1312	1864.5	(75.4)	2220.7	(50.3)	—4.06	(18)	0.01 *

Growth of tumors BN248, BN569, and WR1618 is given as the mean of the individual tgα values (see Materials and methods). BN1312 is given as the mean spleen weight at day of termination, and BN175 is given as mean tumor size (mean of length and width) at day 7.

* statistically significant different.

pacity and hormonal imbalance, due to the stress procedure, in the earlier mentioned studies a retardation in weight gain was noted as well (13, 15). This was confirmed again in a parallel study in non tumor bearing animals, which data is given in Materials and method. This lagging in body weight was apparently not caused by a decreased food intake, as was also shown in the parallel study. In as far the observed small differences in body weight are partly responsible for the established differences in tumor growth (i.e. via hormonal alterations) is hard to assess.

The finding that the type of stress as used in our study gave a decrease of tumor growth seems to contradict the apparent common opinion that stress leads to tumor enhancement. However, stress-induced inhibiting effects on tumor growth were established by others as well, especially in « chronic uncontrollable » stress (22). As most of the data, in which tumor-promoting effects were found, came from experiments in which acute stress was used, the tumor promoting effect in these studies might have been due to the specific characteristics of this type of stress. In a longevity study by us, in which during 150 weeks L-D shift stress was given while spontaneous tumor incidence was determined, differences in spontaneous tumor incidence (although, not statistically significant) were noted, also in favor of the hypothesis that mild chronic stress decreases tumor incidence and prolongs longevity (15).

Apart from affecting tumor growth, stress might influence tumor metastatic pattern as well. In different experimental models, one in which tumor metastasis was mimicked by intravenously injection of tumor cells (i.e. studying tumor take) (18); the other one, in which the number of lung metastasis was established after spontaneous dissemination of the tumor from its inoculation site (24), it was shown that metastases were increased after stress. In our present study using tumor BN175, in a model comparable with the latter mentioned study of Tanemura et al. (24), these results could not be confirmed: on the contrary our data showed a decreased number of metastasis in the L-D shift stressed group. Like mentioned earlier, different types of stress, depending

the specific characteristics of the stressor type, might exhibit different effects on tumor growth and apparently also on tumor metastatic pattern. For this hypothesis confirmation was found in a study of Zimel et al. (29), in which using chronic stress, the formation and growth of metastases was inhibited.

As far as our results concern [in the present experiment as well as in the earlier mentioned parallel study, in which spontaneous tumor incidence was determined (15)], no hazardous effect of a type of mild chronic stress could be detected on tumor growth and metastasis. Still, according to data from other investigators, acute stress may have the opposite effect. The question remains, of course which stress is most relevant to human situations. According to a study of Lundy (16), surgery and anesthesia, which are typical examples of acute stress, had a direct effect on tumor progression particularly with respect to spread, by inducing impairment of immune capability. Uncoped emotional stress, as an example of chronic stress, is held responsible for enhanced tumor incidence, although noxious « life style » habits such as drinking and smoking, which often go hand in hand with the particular characteristics of this specific group of patients, cannot be excluded (3, 17). For shift workers or other groups of workers subject to mild chronic stress some investigators found evidence for enhanced susceptability to sickness, gastro-intestinal and cardiovascular diseases and unspecific health complaints, however, higher tumor risks were not mentioned (2, 12, 23).

Therefore, it seems that mild chronic stress, although detrimental to health, does not lead to enhanced tumor growth or incidence.

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Publication IV

EFFECT OF DIETARY LINOLEIC ACID ON CELLULAR IMMUNE RESPONSE AND TUMOR GROWTH IN RATS

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Effect of dietary linoleic acid on cellular immune response and tumor growth in rats

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Synthetic diets high (17.7 cal.%) and low (3.3 cal.%) in linoleic acid were fed to Brown Norway female rats. One group of animals received a commercially available standard rat chow. Seven weeks after starting the animals on the diet, Concanavalin A stimulation of peripheral blood lymphocytes and a Popliteal Lymph Node Assay was performed. When compared with the rats receiving the standard diets, those on the high and low linoleic acid diets showed decreased cellular immune responses. In another series of experiments, animals were placed on the same three diets and rats from each group were inoculated with tumors one of the following, after 7 weeks: a syngeneic cervical "round cell sarcoma", an urothelial squamous cell carcinoma, a myeloid leukemia and an adrenal cortical carcinoma. Almost no differences were detectable in the growth of the cervical "round cell sarcoma" and the urothelial squamous cell carcinoma. However, there were statistical significant differences in the growth of the leukemia and the adrenal cortical carcinoma, which growth was positive correlated with the decrease in the immune response.

Certain constituents of the diet, especially long chain unsaturated fatty acids such as linoleic acid, have recently been shown to induce immune changes (11, 12, 16, 17, 18, 20, 24). The lymphocyte transformation induced by a variety of mitogens was inhibited *in vitro* by linoleic acid (11, 17, 18, 20). *In vivo* skin graft survival was prolonged after oral and subcutaneous polyunsaturated fatty acid (PUFA) administration (18); although less marked, the same effect was seen in clinical kidney transplantation (16) and on experimental heart and kidney allograft survival (12). Dietary factors are also known to influence tumor growth in many experimental animal models. Tumor growth was enhanced by diets high in PUFA (1, 3, 9, 11).

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Although there is still no direct evidence for this, it seems logical to suppose that the above mentioned immunosuppressive effects of these dietary fats are responsible for this enhanced tumor growth.

A number of studies have been carried out on this subject up to now; however, there is still no conclusive evidence that fat or certain fatty acids are directly or indirectly related to carcinogenesis (3, 17).

Experimental data on the effect of polyunsaturated fatty acids on the immune response and carcinogenesis are not always easy to interpret; for instance, in studies on linoleic acid, this dietary component was given for only short periods of time, often in very high quantities and comparison was sometimes made with groups of animals receiving diets deficient in linoleic acid (19). In the latter cases, the consequences of the pathological conditions of such diets were manife-

sted by extremely low body weights (1).

In the study presented here, the main objective was to determine the cellular immune response of rats receiving diets high and low in linoleic acid for prolonged periods of time in comparison with others receiving a standard diet. In addition, inoculations of immunogenic and non-immunogenic tumors were done in syngeneic rats on the same dietary regimens as those in which the immune response determinations were carried out in order to establish whether such diet induced immune changes could influence tumor growth.

Materials and methods

Experimental design

Determination of the cellular immune responses and tumor growth was carried out in three groups of Brown Norway rats. Group I was placed on a diet high in linoleic acid (C18:2); group II received a low C18:2 diet and group III a commercially available standard rat diet. Seven weeks after the diets were started, Concanavalin A (Con A) stimulation of peripheral blood lymphocytes and a Popliteal Lymph Node Assay (PLNA) were performed. In another series of experiments rats were inoculated with tumors, 7 weeks after starting the diets, of four different characteristics: a cervical « round cell sarcoma », a urothelial squamous cell carcinoma, an adrenal cortical carcinoma and a myeloid leukemia.

Each experimental group consisted of ten animals.

Animals

Highly inbred female Brown Norway (BN) rats, obtained from the University Animal Breeding Centre, were used. At six weeks of age, the animals were randomly selected and distributed into the experimental groups. F1 hybrid animals necessary for the PLNA were bred by mating BN males with inbred Wistar/Rotterdam (WR) females. When the offspring were 7 weeks of age the males were used in the assay. All animals were housed in airconditioned rooms in macrolon cages in groups of 5.

Diets

Linoleic acid was given as a constituent of a total synthetic diet. The diets contained 17.7 (high) and 3.3 (low) calories percent linoleic acid and were prepared by Unilever Research Laboratories, Vlaardingen, Holland. The standard diet given to the rats in group III was a commercially available one (Hope Farms AM II, Woerden, Holland) containing 7.5 calories percent linoleic acid. The fatty acid analysis of the diets used in this experiments is given in Table 1. Although both the standard diet and the one high in C18:2 contained fat with 53% PUFA, the rats of group I received about twice as much PUFA as those of group III because of the diet differences in total fat content.

Food intake of the three different diets was determined in a number of animals. In each diet group intake was established in three cages containing 5 animals each by subtracting the weight of the uneaten food from that of the original amount fed. This procedure was carried out three times, each for a period of two days. Values were converted to consumption per rat per day. The values in the table express the mean plus or minus the standard deviation of nine separate determinations.

The determinations were deliberately not carried out in metabolic cages, as such housing conditions tend to alter food consumption, at least until adaptation has been achieved. Because the high and low C18:2 diets were completely synthetic they did not contain crude fiber and this might explain the small difference in food comsumption as compared with the animals receiving the standard rat chow (Table 1).

The C18:2 diets were freshly prepared weekly in three portions: one for 3 days, the other two for 2 days. To prevent oxidation, the diets were stored at 4° in closed containers into which some nitrogen has been introduced before closure. The animals received the C18:2 diets every Monday, Wednesday and Friday; at the same time the old uneaten food was discarded. Because the Hope Farms food is more stable, uneaten portions of this diet were not discarded but only replenished when necessary.

Leucocytes

Peripheral blood leucocytes were determined in 0.02 ml of heparinized blood, gathered by orbital plexus puncture, using a Sysmex Microcellcounter (Japan). Estimation of the % lymphocytes (necessary for the Con A) was done by making a smear with a drop of heparinized blood and counting 100 cells. The determinations were carried out in groups of 10 animals each.

Concanavalin A stimulation of peripheral blood

$1x10^5$ leucocytes in whole blood (heparinized with 15 IU heparin/ml; blood collected by orbital plexus puncture under light aether anesthesia) were added to each well of Microtitre culture plates (Greiner, W. Germany). RPMI medium (Gibco, Scotland) containing 10% foetal calf serum, 20 µM/ml L-Glutamine, 50 IU/ml of penicillin, 50 µg/ml streptomycin and 10^{-5} β-mercapto-ethanol was added to a volume of 200 µl. Each determination was carried out in quadruplicate. Plates were incubated with 5 µg Con A (Pharmacia, Holland) per well at 37° in a humidified atmosphere

Table 1 - *Fatty acid composition of the diets.*

	Group I C18:2 high n = 10	Group II C18:2 low n = 10	Group III Hope Farms n = 10
Total fat	15.4	15.4	8.2
SFA	2.2 (14%)	7.9 (51%)	1.0 (14%)
MUFA	5.0 (33%)	6.0 (39%)	2.4 (33%)
PUFA	8.2 (53%)	1.5 (10%)	3.8 (53%)
Kcal	411.5	411.5	465.0
Mean food consumption	7.0 ± 0.5	7.0 ± 0.3	8.4 ± 0.5
Mean body weight incr.	60.8 ± 6.0	61.0 ± 5.1	64.9 ± 6.3

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
Total fat and fatty acids in grams/100 g food. In parentheses, percentages of total fatty acids.
Mean food consumption, mean ± S.D. food consumption/rat/day, n = 9.
Mean body weight incr., mean ± S.D. body weight increase over the period of 7-13 weeks of age; n = 20.

of 5% CO2. Harvesting was performed on day 4 of culture. At 16 hours before harvesting, 0.8 μCi of methyl-[^{3}H]-thimidine (with a specific activity of 2 Ci/mmol; Amersham TRA 310) was added to each well. Con A stimulation was expressed as counts/min/culture. The values given were from 10 animals per group.

Popliteal Lymph Node Assay

A local graft-versus-host assay was carried out according to the method described by Levine (14). After local injection of parental cells into F1 hybrid recipients a localized form of graft-versus-host disease will be the result. When cells are injected intradermally in one of the food pads this graft-versus-host reaction takes place in the popliteal lymph node draining the inoculated foot.

In this study the procedure used was as follows: 0.2 ml of whole heparinized blood was taken from the experimental animals (i.e. the diets groups) via orbital puncture and injection into the left foot pad of (BNxWR)F1 hybrids. The F1 hybrids were on standard rat chow. Seven days after injection of the blood into the test animals. the popliteal lymph nodes were excised, cleansed and weighed in tenths of mgs. Graft-versus-host reactivity was measured as mean lymph nodes weight in 10 animals per experimental group.

Tumors

Tumors used consisted of a cervical « round cell sarcoma », a urothelial squamous cell carcinoma, a myeloid leukemia and an adrenal cortical carcinoma. These tumors originated in a colony of aging BN female rats kept at our laboratory, in which they developed spontaneously. Except for the adrenal cortical carcinoma, all tumors were not immunogenic as judged by immunization-challenge type of *in vivo* transplantation tests done according to the method described by Prehn and Main (21). The adrenal cortical carcinoma proved to be immunogenic.

Tumor size was established by weekly determinations of the dimensions of the tumor using a micrometer. The mean of the lenght and the width was taken as a measure of the tumor size. Solid tumor growth was expressed as the mean of the individual regression coefficients (*b*) obtained after calculating the best fitting straight line (linear regression: $y = a + bx$) from the weekly determinations of the tumor size. Growth of the myeloid leukemia was established by weighing the spleen to the nearest milligram after exsanguination of the animal at 18 days after i.v. inoculation of $1x10^6$ tumor cells. Tumors were kept at -70° until use. After one passage of « starting up » in syngeneic rats, tumors were used for inoculation experiments, the solid ones by s.c. implantation of a single piece of approximately 5 mm^3 through a small skin incision in the right side of the rat. The leukemia was injected i.v. in one of the tail veins. All experimental groups consisted of 10 animals each.

Statistical evaluation

Statistically significant differences between 2 groups were established using a Student t-test. The hypothesis that the difference between the means was negligible was rejected at a significance level of 5%.

Results

The results of the cellular immune response determinations after 7 weeks of diet are given in Table 2.

In both the Con A stimulation test and the PLNA, the animals receiving the Hope Farms diet exhibited the best cellular immune capacity as compared with both groups of rats fed on the synthetic diets. On comparing the values for the three diet groups in the Con A stimulation test, group II was found to be significantly lower than groups I and III ($p < 0.01$). However, stimulation by Con A in groups I and III was similar ($p > 0.05$). Although the PLNA values for groups I and II were also lower than those of group III, only the difference between group I and group III was statistically significant ($p < 0.05$).

Differences in either leucocytes or lymphocytes were small and not statistically significant (Table 3).

Table 4 presents tumor growth of the 3 nonimmunogenic tumors and the immunogenic one inoculated into rats receiving the diets high and low in linoleic acid and a standard chow. The cervical « round cell sarcoma » and the urothelial squamous cell carcinoma showed no significant difference in growth in either diet group. However, the other nonimmunogenic tumor, the myeloid leukemia, clearly manifested changes in tumor growth in a sequence parallelling the immune response as determined by the Con A stimulation test. That is to say, group II, the group which gave the poorest result in Con A stimulation, showed the strongest inhibition of leukemia growth, followed by

Table 2 - *Effect of diet on cellular immune response.*

	Group I C18:2 high n = 10	Group II C18:2 low n = 10	Group III Hope Farms n = 10
Con A; counts/ min/culture mean ± S.D.	32,503 ± 9,255	17,519 ± 8,699	43,439 ± 16,212
PLNA; lymphocyte weights in mgs mean ± S.D.	30.9 ± 4.8	32.6 = 6.4	37.0 = 5.7

Con A = Concanavalin A stimulation of peripheral bloqd lymphocytes.
PLNA = Popliteal Lymph Node Assay.

Table 3 - *Effect of diet on peripheral blood leucocytes and percentages of lymphocytes.*

	Group I C18:2 high n = 10	Group II C18:2 low n = 10	Group III Hope Farms n = 10
Leucocytes $x10^6$ mean ± S.D.	9.9 ± 2.8	8.4 ± 2.9	10.3 ± 3.2
% Lymphocytes of leucocytes mean ± S.D.	90.7 ± 3.1	92.8 ± 4.2	92.4 ± 4.3

Table 4 - *Effect of diet on tumor growth.*

	Group I C18:2 high n = 10	Group II C18:2 low n = 10	Group III Hope Farms n = 10
Cervical round cell sarcoma mean growth ± S.D.	10.28 ± 1.16	10.22 ± 0.97	11.45 ± 1.59
Urothelial squamous cell carcinoma mean growth ± S.D.	5.06 ± 1.10	5.02 ± 0.93	4.83 ± 0.57
Adrenal cortical carcinoma mean growth ± S.D.	2.84 ± 0.73	3.80 ± 0.79	6.27 ± 1.38
Myeloid leukemia spleen weight mean ± S.D.	1196.8 ± 208.4	946.8 ± 151.1	1752.5 ± 347.7

Tumor growth is expressed as the regression coefficient of the straight line resulting from the weekly determinations of the tumor size.

group I and III (Group I vs Group II, $p < 0.01$; Group I vs Group III, $p < 0.001$; Group II vs Group III, $p < 0.001$). The adrenal cortical carcinoma, the one immunogenic tumor model used in this study, also showed differences in growth pattern. Again, tumor growth was most prominent in group III, the group of animals receiving the Hope Farms diet; tumor growth in group I was slightly more delayed than that of group II (Group I vs Group II, $p < 0.05$; Group I vs Group III, $p < 0.01$; Group II vs Group III, $p > 0.05$).

Discussion

In an earlier study, we reported that a synthetic diet high in fat consisting primarily of PUFA prolonged kidney allograft survival in rats (12); however, a diet containing the same amount of fat in which sunflower oil was replaced by palm oil was only slightly less effective.

In this study, more or less the same diets were provided to investigate the immune response and tumor growth. The finding that diets high in fat exerted an immunosuppressive effect on the cellular immune response was also observed in a number of other investigations (3, 11, 18, 24). However, in the literature, especially the PUFA level of the diet was held responsible for the immunosuppressive effect of high fat diets (1, 11, 16). Even immunopotentiating effects were described when diets low or deficient in C18:2 were given (18). In our study, no indication for such an effect of the low C18:2 diet was found. We think that this discrepancy is in some way due to the pathological effects of diets very low or deficient in C18:2 as used by others (8). Some support for this statement is the finding of Hillyard and Abraham (7), who in a study on the growth promoting effect of dietary PUFA on transplantable mouse adenocarcinoma gave increasing amounts of C18:2 and found that addition of as

little as 0.1% of C18:2 to the fat free diet was enough to abolish the tumor growth inhibiting effect of such a diet.

In addition to the direct consequences of the immuno-inhibiting effects of high fat diets on, for instance, virus or bacterial susceptibility (17), these is substantial evidence that high fat diets also enhance tumor growth (1, 3, 17). Most investigators are unanimous in their opinion that high fat diets enhance tumor growth; however, the role of PUFA in this phenomenon is subject to much debate (1, 3, 4, 22).

In our study on tumor growth, no enhanced growth was observed in either the syngeneic nonimmunogenic tumors or the immunogenic tumor model.

Although in our leukemia study a significantly higher spleen weight was found in the animals receiving a high PUFA diet as compared with the low PUFA diet [an observation earlier shown in a study on dietary fat saturation on the survival of mice with leukemia (2)], this difference in tumor growth was surpassed by far by leukemia growth in the animals of Group III, which were on standard diet. Because the PUFA content of this diet results in plasma PUFA percentages somewhere in between those in Groups I and II (13), the PUFA content as such cannot be held responsible for the differences in tumor growth in the leukemia experiment.

Moreover, the finding that a high PUFA diet enhanced tumor growth as compared with a low PUFA diet could not be confirmed in the other three tumor models. From the results of the adrenal cortical carcinoma experiment, even the opposite conclusion could be drawn.

Although a (positive!) correlation was shown between immunosuppression and adrenal cortical carcinoma growth, this still does not necessarily imply that immunosuppression as such is responsible for the observed phenomena.

Changes in cell membrane fluidity or other disturbances of the cell kinetics, as demonstrated after changing the fat composition of the media in cell culture studies (5, 6, 22, 23, 25) may be responsible for changes in mitotic activity *in vivo* situations as well as the decrease in blastogenesis in the Con A stimulation test. If this is the case, the observed depressed immune function would wrongly be considered as the cause of the changes in tumor growth, while both tumor growth and the decreased cellular immune response as determined by the Con A stimulation test are expressions of the same phenomenon. Which means that, in diet studies, we probably have to look for other criteria to measure immune capacity as well, preferably in *in vivo* situations, such as for instance by determining allograft survival or bacterial susceptibility.

The results obtained strongly suggest that no direct correlation exists between immunosuppression, as been measured by cellular immune response assays, and tumor growth. The reason for the differences in tumor growth, as been found in our study, remains unclear, but may be explained by other mechanisms. For instance dietary changes in tumor prostaglandin synthesis capacity (10) or alterations in cell membrane permeability (23) may have been involved.

This study proved once again, that dietary factors can react very differently in different tumor systems. This leads us to the conclusion that, whenever dietary effects on tumor growth are judged, this should preferably be done on the basis of the results of more than one tumor model, as the specific characteristics of a given tumor model are apparently of major importance in the results obtained.

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Publication V

SPONTANEOUS TUMOR INCIDENCE IN FEMALE BROWN NORWAY RATS AFTER LIFELONG DIETS HIGH AND LOW IN LINOLEIC ACID

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Spontaneous Tumor Incidence in Female Brown Norway Rats After Lifelong Diets High and Low in Linoleic Acid [1,2]

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ABSTRACT—High linoleic acid (C18:2) (group I; 17.7 cal%) and low C18:2 (group II; 3.3 cal%) diets were given to groups of inbred Brown Norway virgin female rats (100 animals/group), during their whole life-span. A total of 140 tumors were found in group I and 123 tumors in group II; the median survival times of the 2 groups were 124.2 and 118.5 weeks, respectively. Total spontaneous tumor incidence and median survival times were not significantly different. However, significant differences were found in the incidences of some specific tumors: The numbers of reticuloendothelial tumors and adrenocortical carcinomas were significantly higher in the group of animals receiving the low-C18:2 diet. A high incidence of tumor multiplicity, however, resulted in a significantly greater number of mammary tumors in the high-C18:2 diet group.—JNCI 1985; 74:529-536.

Numerous epidemiologic studies demonstrated that nutrition and diet are related to cancer incidence (*1-3*). Although specific direct-acting carcinogens in the diet may play a role, the relationship between diet and cancer is probably more complex. An important role has been ascribed to caloric intake and the type of fat in the diet; especially fat and particularly the amount of PUFA have received much attention.

In experimental animal studies, dietary fats high in PUFA are reported to be more effective in the promotion of chemically induced tumors than diets low in PUFA (*4-6*). In the interpretation of such studies one has to keep in mind that nutrient deficiencies may lead to biochemical alterations that promote neoplastic processes (*7*). Therefore, studies in which the effect of a high-PUFA diet on tumor promotion is investigated, the control group should receive enough PUFA to prevent disorders that would result from PUFA deficiency, otherwise the experiments may lead to false-positive results. To illustrate this statement, the addition of as little as 0.1% of C18:2 to a PUFA-deficient diet was sufficient to abolish the tumor-growth-inhibiting effect of this diet (*8*).

Many studies investigating dietary factors and their influence on carcinogenesis were conducted with the use of chemically induced tumors, which are, in contrast to spontaneous tumors, uniformly immunogenic (*9*). Since diets that contain high amounts of C18:2 were often found to be able to suppress the immune system (*10, 11*), the growth of immunogenic tumors may be particularly influenced by dietary factors.

Aside from these considerations, which might explain, at least in part, the effect of high-PUFA diets on tumor growth, it appeared that high-PUFA diets did not always enhance carcinogenesis (*12, 13*). For instance, in experiments with rapeseed oil diet, which contains a high level of PUFA, mammary carcinogenesis was not enhanced (*12*).

The present study was designed to determine whether diets high (17.7 cal%) and low (3.3 cal%) in C18:2, quantities of C18:2, which are well within the limits of the dietary standards for humans and animals (*14, 15*), would exhibit a difference in the occurrence of spontaneous tumors. The experiments involved the feeding of the test diets exclusively to BN rats throughout their lifetime; no carcinogens (chemical or otherwise) were given. The "normal" cancer incidence of the BN female rat, as used in the present study, has been described in a number of comprehensive studies in which this rat strain was subject to gerontological research (*16*). Aside from determining the body weight, handling of the animals was minimized; handling was also standardized to prevent possible interactions of variables other than the diet on the outcome of the results.

MATERIALS AND METHODS

Experimental animals.—Two groups of 100 inbred female BN rats were used. These rats were the offspring of the BN animals used in a previous study on retired breeders (*17*). Second and third litter female rats were used exclusively from breeder animals whose age variation was held strictly within limits (mean age: 25.5 wk old). The animals were at their 17th generation of inbreeding at the time of the experiment. Immediately

ABBREVIATIONS USED: C18:2=Linoleic acid; MST=median survival time; PUFA=polyunsaturated fatty acids.

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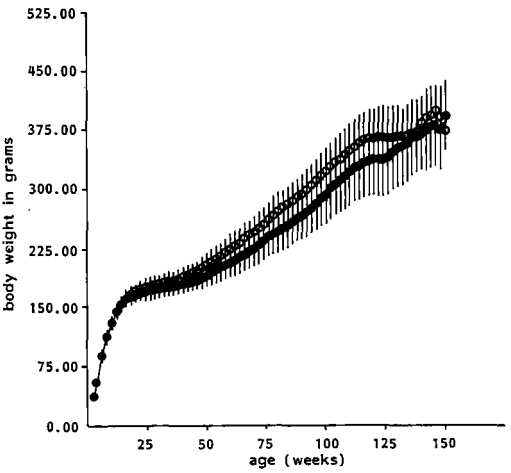
after weaning at 3 weeks, the animals were divided equally in groups and fed the test diets. All animals were housed in Makrolon cages on presterilized woodchips (Woody-Clean 3/15; Broekman Instituut, B.V., Helmond, The Netherlands) in groups of 5 in one air-conditioned animal room with controlled day-night light cycle. To prevent possible effects of dominating animals on e.g., food consumption, the rats were regrouped every 2 weeks; the animals were also rearranged in groups of 5, when necessary. The location of the cage in the animal room was changed every 2 months. At least twice daily, the cage was carefully checked for sick and dead animals without handling them. Animals suspected to be sick were removed and housed individually or sacrificed when moribund. Animals with substantial tumor growth that impeded eating and drinking habits were also killed.

The protocol of sick and dead animals was the same as described elsewhere (*17*). Body weight was determined fortnightly and was used as a criterion for the condition of the animals and for comparison of growth of animals on the two dietary regimens.

Diets.—The diets contained 17.7 (high) and 3.3 (low) calorie % C18:2 given as a constituent of a total synthetic diet prepared by Unilever Research Laboratories, Vlaardingen, The Netherlands. The diets were the same as those used in previous studies on diets and tumor inoculation (*13, 18*). The fatty acid composition of the diets, determined by gas chromatography is given in table 1. This table shows that the diets were isocaloric and were identical for all constituents except the fatty acid composition. The diets were freshly prepared weekly in three portions: one portion for a 3-day period, the other two for a 2-day period. The animals received these diets ad libitum every Monday, Wednesday, and Friday; at the same time the leftover food was discarded.



TEXT-FIGURE 1.—Comparison of the body wt increase in rat groups I and II, fed ●, 17.7 cal% C18:2; and ○, 3.3 cal% C18:2. *Vertical bars* represent the standard deviations. Differences in body wt were statistically significant after 20 weeks.

TABLE 1.—*Characteristics of the study diets*

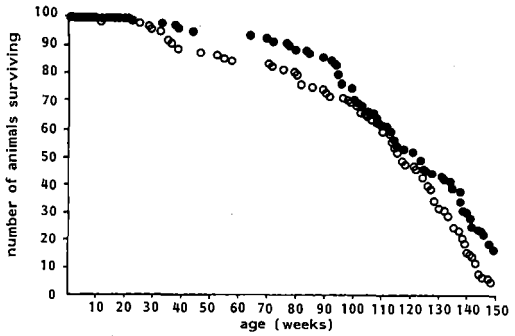
Diet compound	Group I[c]	Group II
Composition of the diets in caloric percentage		
Albumin	23	23
Total fat	35	35
C18:2 (included in total fat)	17.3	3.3
Carbohydrates	42	42
Distribution, g (1,000 kcal)		
Casein	64.111	64.111
Palm oil	—	37.634
Corn oil	28.000	—
Sunflower oil	9.634	—
Starch	120.000	120.000
Minerals (Cas. '73)[a]	5.354	5.354
Vitamins (Vit. '70)[b]	1.000	1.000
Cellulose	15.000	15.000
Total	243.099	243.099
Composition of fatty acids, %[c]		
Palmitic acid	11.1	45.8
Stearic acid	3.3	4.3
Oleic acid	32.4	38.2
Linoleic acid	50.7	9.5
Linolenic acid	0.9	0.2
Arachidonic acid	1.6	2.0

[a] In mg/1,000 kcal: potassium chloride, 350; secondary magnesium phosphate, 956; primary potassium phosphate, 475; potassium bicarbonate, 719; calcium carbonate, 2014; trisodium citrate 2 H_2O, 711; manganese sulfate, 67.8; ferric citrate, 43.9; copper citrate, 4.7; zinc citrate, 12.5; potassium iodate, 0.07.

[b] In mg/1,000 kcal: choline (50%), 500; vitamin E (250 IU/g), 80; calcium silicate, 50; myoinositol, 25; vitamin B_{12} (1,000 mg/kg), 5; vitamin A (325 IU/mg), 7.7; nicotinic acid, 5; (calcium)-pantothenic acid, 5; riboflavin, 1.5; thiamine mononitrate, 1.5; vitamin D (80 IU/mg), 3.125; vitamin K_3 (22.7%), 1; pyridoxine, 0.5; folic acid, 0.25; biotin, 0.05; saccharose, 314.375.

[c] Established by gas chromatography.

Autopsy.—Autopsies were conducted according to the protocol described in one of our previous longevity studies (*17*). Each organ system was examined, and macroscopic findings were recorded. Thymus, adrenal gland, and spleen weights were determined as measures of immune capacity. Routine microscopic examinations were performed on samples of liver, spleen, mesenteric lymph nodes, peripheral lymph nodes, kidneys, adrenal glands, stomach, duodenum, ileum, cecum, colon, uterus-cervix-vagina, pancreas, pituitary gland, thyroid gland, thymus, heart, lungs, aorta, muscle, ovaries, mammary gland, and bone marrow. Other organs were only examined when suspected of neoplasms. Tissues were fixed in 4% buffered formaldehyde and after processing in an Autotechnicon apparatus, embedded in paraffin. Sections of at least 5 μm-thick were stained with hematoxylin and eosin. Other stains were used when required by our pathologist (P. Z.). All materials were screened at least twice by the pathologist; in a number of cases additional expertise was requested from other experienced pathologists. Cardiac ischemia was scored when the heart muscle showed areas where "waviness,"



TEXT-FIGURE 2.—Comparison of the actuarial survival of groups I and II rats, receiving ●, 17.7 cal% C18:2; and ○, 3.3 cal% C18:2. Each group originally consisted of 100 animals; diets were given during the whole life-span.

contraction bands, nuclear pyknosis, and deep eosinophilia of the cytoplasma were present.

Statistical evaluation.—The Peto analysis (*19*), which is a chi-square analysis adapted to evaluate cumulative incidence data, was used. In the results, the calculated values are given as *G*-values with their corresponding *P*-values. The correlations between body weight and tumor incidence were calculated with the use of Fisher's exact probability test. Finally, a Mann-Whitney analysis, a parameter-free test was used to compare the differences in mean age (*Z*-values). Differences were considered to be statistically significant when $P \leq .05$.

RESULTS

Husbandry and Body Weights

Husbandry data of all animals, i.e., litter size, weight at weaning, age of the mother at litter, week of birth, and mortality in the litter were related to survival and tumor incidence. None of these comparisons showed significant correlation. No correlation was found either in mother and daughter relationships for tumor and nontumor incidence: among the 35 animals with mammary tumors in the study, only 1 mother was found with the same type of tumor as its daughter. Thus all possible effects from variables other than C18:2 content of the diets on the final results were excluded.

As a direct result of the difference in the diets a difference occurred in the mean body weights of the groups. Despite the fact that the two diets were isocaloric, the body weight gain of the 2 groups started to diverge early in the experiments (text-fig. 1). By 20 weeks of age, a statistically significant difference in body weights was found (mean±SE: group I, 166.0±0.9; group II, 169.3± 0.9). From this time on, the mean body weight of animals in group II was higher than that in group I. The maximum difference in mean body weights (20 g) was reached at about 100 weeks, after which the weekly weight gain was almost equal. The differences in body weights in the 2 groups, however, remained statistically significant in the ensuing period.

Body weights of group I and group II animals at termination, when killed or found dead, were not significantly different. There were no differences either in the mean weights of the thymus, spleen, and adrenal glands determined at autopsy.

It is obvious that differences in body weight may have consequences on survival or tumor incidence. For survival in general the body weight does not seem to be of importance. However, the incidence of mammary tumors is positively correlated with body weight at 40 weeks $G=5.05$; $P<.05$). We did not find any correlations of body weight at other time intervals with either mammary tumors or with other tumor or nontumor processes.

From the 100 animals in each dietary group, 17 and 5 animals survived the 150-week observation period in group I and II, respectively. MST of group I and II were 124.2 and 118.5 weeks, respectively, a statistically not significant difference ($Z=-1.47$; $P=.14$).

The data of all animals were included in the actuarial survival curve (text-fig. 2). For all other evaluations, 2 animals in group I and 4 animals in group II were excluded. Due to autolysis and cannibalism these animals could not be examined either macroscopically or microscopically.

TABLE 2.—*Incidence of nontumor processes and mean ages of rats*[a]

Nontumor process	Mean age, wk, and incidence ± SE				Mann-Whitney analysis of mean age		Peto analysis of nontumor process incidence	
	Group I	*n*	Group II	*n*	*Z*-value	*P*-value	*G*-value	*P*-value
Uterine infection	101.2±4.4	18	109.7±6.1	17	−0.97	.33	0.05	.80
Hydronephrosis	128.5±4.2	31	128.0±2.7	35	−1.07	.29	2.22	.15
Biliary cysts	130.1±2.7	44	128.3±3.0	30	−0.71	.47	0.40	.55
Stomach bezoars	127.6±2.5	70	122.3±3.5	52	−1.21	.22	0.16	.68
Pancreatic atrophy	134.3±4.0	23	125.9±5.9	13	−1.76	.08	0.73	.40
Mammary hyperplasia	128.5±5.1	19	131.6±4.9	16	−0.25	.80	0.09	.75
Cardiac ischemia	141.7±5.2	9	134.9±2.4	25	−2.11	.03[b]	13.28	.01[b]
Bronchopneumonia	71.3±29.9	3	83.4±13.0	9	−0.09	.93	3.57	.06

[a] Group I received 17.7 cal% C18:2 in diet; group II received 3.3 cal%.
[b] Statistically significant; $P<.05$.

Mortality

Mortality of the rats in group II was higher than that in group I, particularly in the first 90 weeks of the experiment (text-fig. 2). After this period of high mortality, predominantly due to infectious diseases, mortality from tumor processes gradually increased in an almost identical pattern in both groups. The large number of animals dying in the first year of the experiment is the result of a high incidence of uterine infections, bronchopneumonia, and other nontumor processes (table 2).

Animals dying from genital tract disease died mostly without any clinical symptoms, e.g., without loss of body weight. Macroscopically, the uterus appeared swollen, filled with a pale viscous fluid. Microscopically, neutrophylic exudate was visible in the oviduct lumen and polymorphonuclear leucocyte infiltration in the submucosa, indicating severe endometritis. This disease, described by Cassell et al. (*20*), is caused by Mycoplasma pulmonis and has been demonstrated in 30–40% of the female rats in a given colony, although certainly not all these animals showed clinical signs of disease. The disease progresses much more rapidly in older animals and in animals with an inferior immune system (*20*).

Animals dying from bronchopneumonia were sometimes ill for a few days, with obvious difficulties in breathing. The course of this disease was also very rapid and death could not always be predicted. Culture of heart blood taken from a number of these animals revealed infections of *E. coli* (4 rats), *Enterococcus* (2 rats), *Pseudomonas* (2 rats), and *Pasteurella pneumotropica* (2 rats).

Due to the rapid course of these illnesses, a sick or dying animal was not always recognized as such; it was then found dead at one of the daily inspections. Organs from such animals were often not available for examination. Since most of these organs were from rats dying in the first two-thirds period of the experiment when tumor incidence was still small, the unavailability of these organs for microscopic or macroscopic examination did not influence significantly the analyses of the tumor incidence data. Moreover, in the Peto analyses (*19*) of differences in incidence between groups I and II, the missing organs were included in the statistical analysis. Since a large number of thyroids were missing for evaluation, 16 in group I and 17 in group II, respectively, the results of tumor or nontumor processes for this specific organ may have been affected.

Hydronephrosis, biliary cysts, and stomach bezoars, which occurred at a very high frequency, did not show any significant difference, either in incidence (Peto analyses) or in relation to mean age (Mann-Whitney analyses). Statistically significant differences were observed only in the incidence of cardiac ischemic lesions and bronchopneumonia. These processes were significantly more often encountered in group II. The mean age of animals with cardiac ischemic lesions in group II was also statistically significantly lower compared to that of group I animals.

Spontaneous tumor incidence showed great variability

TABLE 3.—*Incidence pattern of spontaneously occurring tumors*[a]

Sites and types of tumors	Group I		Group II	
	No. of rats with tumors	MST	No. of rats with tumors	MST
Pituitary gland				
Carcinoma	2	144	—	
Adenoma	29	139	14[b]	138
Adrenal gland				
Cortical carcinoma	7	135	15	129
Cortical adenoma	4	150	5	136
Medullary pheochromocytoma	4	142	3	145
Pancreas endocrine				
Islet cell carcinoma	—		3	129
Islet cell adenoma	29[b]	146	27[b]	133
Pancreas exocrine				
Adenocarcinoma	1	149		
Fibrosarcoma	—		1	81
Thyroid gland				
Medullary carcinoma	7	148	3	140
Ovaries				
Papillary cyst adenoma	—		1	131
Mammary gland				
Adenocarcinoma	2	134	1	100
Adenofibroma	19[b]	144	14	132
Fibroma	1	150	—	
Brain				
Meningioma	1	33	—	
Oligodendroglioma	1	107	—	
Schwann cell tumor	1	116	—	
Granular cell myoblastoma	1	142	4	114
Cervix and vagina				
Squamous cell carcinoma	2	129	—	
Leiomyosarcoma	2	84	3	91
Oviduct and uterus				
Leiomyosarcoma	3	116	2	104
Leiomyoma	3	138	2	131
Hemangioendothelioma	2	127	2	99
Liver				
Hepatocellular carcinoma	3	139	—	
Hemangioendothelioma	2	107	—	
Gastrointestinal tract				
Squamous cell carcinoma	1	108	1	111
Leiomyosarcoma	2	117		
Leiomyoma	3	135	2	127
Adenoma	—		1	150
Fibroma	1	148	1	136
Lipoma	1	150	—	
Papilloma	1	150	—	
Skin and subcutaneous tissue				
Melanoma	1	124	3	143
Squamous cell carcinoma	1	150	—	
Lipoma	—		1	106
Salivary gland				
Adenocarcinoma	1	95	—	
Lungs				
Adenoma	—		1	133
Kidney				
Transitional cell carcinoma	—		1	139
Lymphoreticular tumor				
Histiocytic sarcoma	—		2	119
Myelomonocytic leukemia	—		4	122
Lymphoblastoma	2	117	3	114

[a] Group I received 17.7 cal% C18:2 in diet; group II received 3.3 cal%.
[b] This figure includes the No. of animals in which >1 tumor was found. *See* also table 6.

TABLE 4.—*Incidence of specific tumor processes and mean age*[a]

Tumors	Mean age, wk, and tumor incidence ± SE				Mann-Whitney analysis of mean age		Peto analysis of tumor incidence	
	Group I	*n*	Group II	*n*	*Z*-value	*P*-value	*G*-value	*P*-value
Tumor (general)	128.4±2.7	71	122.9±2.6	75	−1.86	.06	2.83	.09
Mammary glands	139.2±3.2	20	115.6±8.2	15	−2.59	.01[b]	0.00	.90
Adrenal cortex	133.4±6.4	11	126.3±3.9	18	−1.16	.25	3.89	.05[b]
Pituitary gland	133.4±3.4	31	135.2±3.1	14	−0.42	.68	2.61	.12
Pancreatic islets	140.2±2.3	29	132.8±2.4	30	−2.29	.02[b]	1.53	.22
Thyroid gland	144.4±2.7	7	140.3±2.6	3	−1.04	.29	0.50	.47
Lymphoreticular system	117.0±21.0	2	119.2±4.8	9	−0.24	.81	4.48	.04[b]

[a] Group I received 17.7 cal% C18:2 in diet; group II received 3.3 cal%.
[b] Statistically significant; $P<.05$.

with regard to sites, as well as tumor types (table 3). In particular, tumors of the exocrine organs were seen with high frequency. For a number of important tumor sites, mean age and incidence are given in a separate table (table 4). Neither the total number of tumors nor the mean survival time of the whole group of animals with tumors was statistically significantly different when the 2 experimental groups were compared. However, significant differences in tumor incidences were found for 2 tumor types: for adrenocortical carcinomas and tumors of the lymphoreticular system (leukemia and lymphoblastic sarcomas). For both tumor types the incidence in group II (C18:2-low) was significantly higher compared with the rats that received the C18:2-high diet. Although the incidences of Langerhans islet tumors or mammary gland tumors were not significantly different (Peto analysis), the mean age at which these tumors were found was significantly higher in group I. The differences in tumor mortality from pituitary gland adenomas, thyroid carcinomas, and mammary tumors, which seemed to be substantially more numerous in group I, were caused only by the differences in survival in the last year of the experiment. When such differences were corrected for in the Peto analysis, they appeared to be statistically not significant.

The mean numbers of tumors per rat in groups I and II were 2.1 and 1.6, respectively (table 5). This difference was not statistically significant either, because of the smaller number of animals surviving until the final part of the experiment in group II, a period in which animals were more often found dying with more than 1 tumor than earlier in the experiment. A Peto analysis done on tumor multiplicity (>1 tumor/rat) confirmed this finding (G-value=0.74; P-value=.40). A gradual increase in the number of tumors was noted in both groups. In the first 100 weeks of the study only 33% of the animals in group I and 37% in group II were found dead with only 1 tumor in most cases. In the last 50 weeks of the experiment this pattern changed considerably; in group I and II, 73 and 91% of the remaining animals, respectively, were found with 1 or more tumors.

Animals were found not only with tumors at different anatomic sites, but also in some cases with more than 1 tumor at the same anatomic site (table 6). In particular, this was the case with group I animals given the high-C18:2 diet. The total number of mammary tumors in this group was almost twice as high as that in group II. A Peto analysis showed that this difference was statistically significant (G-value=4.54; P-value=.04).

TABLE 5.—*Number of tumors in tumor-bearing rats*[a]

Age, wk	Total No. of rats dying	No. of rats dying with tumor	No. of rats with the following No. of tumors:							No. of tumors	
			0	1	2	3	4	5	6	Total	Mean[b]
Group I											
0–10	0	0									
11–20	0	0									
21–30	1	0	1								
31–40	3	1	2	1						1	1.0
41–50	1	0	1								
51–60	0	0									
61–70	2	0	2								
71–80	4	1	3	0	1					2	2.0
81–90	3	1	2	1						1	1.0
91–100	11	5	6	3	2					7	1.4
101–110	13	8	5	6	2					10	1.3
111–120	9	5	4	3	2					7	1.4
121–130	9	9	0	3	5	1				16	1.8
131–140	14	13	1	5	1	5	0	2		32	2.5
141–150	30	28	2	8	5	9	3	2	1	73	2.6
Totals	100	71	29	30	18	15	3	4	1	149	2.1
Group II											
0–10	0	0									
11–20	1	0	1								
21–30	3	0	3								
31–40	7	0	7								
41–50	1	0	1								
51–60	3	2	1	2						2	1.0
61–70	0	0									
71–80	4	2	2	2						2	1.0
81–90	6	3	3	3						3	1.0
91–100	5	4	1	4						4	1.0
101–110	8	7	1	3	3	1				12	1.7
111–120	14	11	3	7	3	1				16	1.5
121–130	13	12	1	6	5	1				19	1.6
131–140	16	16	0	6	7	3				29	1.8
141–150	19	18	1	8	4	4	2			36	2.0
Totals	100	75	25	41	22	10	2			123	1.6

[a] Group I received 17.7 cal% C18:2 in diet; group II received 3.3 cal%.
[b] Calculated by dividing the No. of tumors by the No. of tumor-bearing rats.

TABLE 6.—*Number of rats with more than 1 tumor at the same anatomic site*[a,b]

Anatomic site	No. of group I rats with No. of tumors:			Total No. of tumors	No. of group II rats with No. of tumors:			Total No. of tumors
	1	2	3		1	2	3	
Mammary glands	14	5	1	27	15	0	0	15
Langerhans islets	25	4	0	33	27	3	0	33
Pituitary gland	31	0	0	31	13	1	0	15
Adrenal gland	11	0	0	11	18	2	0	22

[a] Group I received 17.7 cal% C18:2 in diet; group II received 3.3 cal%.
[b] The difference in mammary tumor multiplicity between group I and group II was statistically significant; $P<.05$.

Correlations were observed between certain endocrine tumors and mammary tumors in groups I and II. For instance, correlations of mammary tumors with either pituitary gland or Langerhans islet tumors were seen with high frequency in group I (48.2 and 40.7%, resp.), but not in group II (20.0 and 13.3%, resp.). This observation cannot be ascribed entirely to differences in total tumor incidence, certainly not with respect to the Langerhans islet tumors, because the numbers of this tumor are not substantially different in the 2 groups. Combination of mammary tumors with adrenal gland or thyroid gland tumors were seen less frequently and seemed to be coincidental.

The only observed correlation of some importance between a tumor process and a nontumor process was the combination of pituitary gland adenoma and mammary hyperplasia. Pituitary gland tumor was associated with mammary hyperplasia in 12 of 31 animals in group I and in 4 of 14 animals in group II. The diagnosis of most of the pituitary gland tumors, chromophobic adenoma, is in agreement with these findings.

The number of animals with distant metastases is given in table 7. Because of their small number, no conclusions could be drawn on the effect of the diet on tumor metastatic behavior. However, the results show that with respect to adrenocortical carcinomas, the only group of tumors with a high metastatic rate, no influence of either diet on metastatic behavior can be established.

TABLE 7.—*Number of tumors found with distant metastases*[a,b]

Tumor site	Type of tumor	Total No. of tumors/ No. tumors with metastases	
		Group I	Group II
Cervix	Squamous cell carcinoma	2/1	—
Adrenal gland	Cortical carcinoma	7/4	15/7
Pancreas	Langerhans islet cell carcinoma	—	3/2
Pancreas	Fibrosarcoma	—	1/1
Skin	Melanoma	1/0	3/1

[a] Group I received 17.7 cal% C18:2 in diet; group II received 3.3 cal%.
[b] The tumors were metastasized to the pancreas (cervix, squamous cell carcinoma), lungs (pancreas, fibrosarcoma; adrenal gland, cortical carcinomas), liver (adrenal gland, cortical carcinomas), brain (melanoma), and mesenterium (Langerhans islet cell carcinoma).

DISCUSSION

For the study of the influence of dietary factors on carcinogenesis, most investigators predominantly used transplantable tumors or tumors induced by carcinogens. Only a few reports describe mice, hamsters, or rats used in longevity studies (*16, 20, 21*). The most important advantage of longevity studies is that the influence of a certain dietary factor on carcinogenesis is established under "normal" environmental conditions. The disadvantage is of course the long duration of these experiments, which leaves room for a number of extrinsic or intrinsic factors to cause unwanted variability of the results. A number of such factors were carefully controlled in the present study, such as the age of the mother, the litter parity, and the conditions in the animal room. Handling of the animals may certainly influence their condition or survival. Stress, the direct result of handling, is considered to be an important factor in immune response changes (*22*) and in carcinogenesis (*23*). Therefore, handling of the animals was restricted to weighing and the necessary changing of bedding and drinking water. Because blood sampling or anesthesia were considered too much stress, immune changes and endocrine organ function were evaluated only in parallel studies in other rats of the same inbred strain. These studies intended to establish a correlation between diet-induced immune changes and tumor growth (*13*).

The test diets used in the present study contain physiologic quantities of essential fatty acids; however, the total amount of fat (15.4 g%) has to be considered high when compared with a commercial rat chow (the standard laboratory diet in our Institute, Hope Farms, contains 8.2 g% of fat). In a longevity study with the same BN rat strain fed the Hope Farm diet, Burek (*16*) observed the same general pattern of spontaneous tumor incidence seen in our study, although there were some differences in the number of tumors at some anatomic sites.

In the classical studies of Tannenbaum in rats (*24*), as well as in epidemiological studies in man (*25*), the differences observed in tumor incidence correlated with body weight. Therefore, a possible effect of the body weight had to be considered. Whereas at 40 weeks more fat animals were found with tumor than thin animals, this correlation between body weight and tumor incidence disappeared at later times in the experiment. The differences in tumor incidence, and more specifically in

mammary tumor incidence, between fat and thin animals at 40 weeks were not restricted to one or the other group; they were present in both dietary groups. The finding that weight at a certain age may have consequences on survival or on tumor incidence has been observed in other studies as well (*26*).

To judge the observed differences in tumor incidence, two different aspects of the results should be considered: the age of the animal at tumor occurrence and the incidence for a particular tumor type. With regard to the first aspect, mammary tumor-bearing rats and rats with a Langerhans islet tumor were found significantly earlier among the animals of group II. Because a direct relationship exists between the age and body weight of rats, not the age at termination but, more importantly, the body weight may be correlated with the occurrence of a certain type of cancer. With respect to mammary tumor incidence, the mean body weight at termination of these animals was significantly less in group II than that in group I. However, this was not the case for Langerhans islet tumor-bearing rats. This observation means that the early occurrence of mammary tumors in group II is not the result of the higher mean body weights of the animals in this group. For Langerhans islet adenomas such a correlation cannot be excluded.

The early appearance of the Langerhans islet adenomas in group II may have been caused by the higher activity of the islets established by the significantly increased insulin levels observed in the parallel studies (*27*).

The mechanism responsible for the differences in adrenal gland and lymphoreticular tumor incidence is even more difficult to explain. The incidence of both tumors was significantly higher in animals on the low C18:2 diet; therefore the same reasons outlined above may hold for these differences as well. It is not difficult to make this assumption for the adrenal gland tumors; a correlation between certain endocrine organ functions and adrenal gland tumors was established by others as well (*28*). In an experiment with Syrian hamsters, the incidence of adrenocortical carcinomas was significantly higher in the high-fat diet group (*21*).

With regard to the differences in leukemia incidence, a role of the endocrine system is unlikely. C18:2 seems to be protective with respect to this tumor type: The incidence in group II (9%) is comparable to what Burek (*16*) found in his study (7%) in animals fed standard rat chow.

The number of tumors per rat increases according to the age of the animals. We saw a tendency for the number of tumors per rat in group I to be higher than that in group II. However, except for mammary tumors, this difference in tumor multiplicity was not statistically significant. The importance of the observed mammary tumor multiplicity is difficult to assess. In studies with chemical carcinogens, tumor multiplicity is used as a criterion for the efficacy of the treatment (*5, 6*); this seems logical because the carcinogen has a direct effect on certain cell types that are transformed to tumor cells. Tumor multiplicity is then a useful parameter to determine the efficacy of the antitumor therapy. However, it would seem extreme to consider C18:2 a chemical carcinogen with a direct effect on mammary tumor carcinogenesis, although there is some support for this idea. Wicha and others (*29*) studied the action of C18:2 in culture medium and found enhanced growth of neoplastic and normal mammary tissue. The classical work of Prehn (*9*) on tumor immunogenicity also fits in this picture: He denies the spontaneous etiology of tumors and considers such tumors as the results of low levels of oncogens. Prostaglandins, which are metabolic end products of C18:2 were also found to play a direct role in mammary tumorigenesis (*30*).

A high number of mammary tumors were found together with pituitary gland tumors and Langerhans islet tumors in group I but not in group II. This finding could imply that with increasing quantities of C18:2 a higher number of mammary tumors appear, induced via specific endocrine stimulation. That hormones play a role in the etiology of mammary tumors is widely accepted; evidence for this interaction was found in epidemiologic as well as in animal studies (*31, 32*).

The results of the present study show that diets high in PUFA (17.7 cal%) are generally not carcinogenic. This finding is corroborated by the results from the parallel studies, in which the same diets were investigated on transmissible tumors (*13*). However, a possible effect of the diet high in C18:2 on the etiology of mammary tumors cannot be denied. That diets high in PUFA are relatively protective against cardiovascular disease was confirmed in this presentation by a significantly lower incidence of ischemic heart lesions in the rats receiving this diet. This seems to be an important reason for the inclusion of high quantities of PUFA in modern human diet. Although no low-fat diets were given, a prudent comparison with data from other studies and our own data suggests that the quantity of fat is more important for enhanced tumor incidence than the distribution of fatty acids.

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Publication VI

INFLUENCE OF THE LINOLEIC ACID CONTENT OF THE DIET ON TUMOR GROWTH IN TRANSPLANTABLE RAT TUMOR MODELS

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Influence of the Linoleic Acid Content of the Diet on Tumor Growth in Transplantable Rat Tumor Models

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Key Words. Linoleic acid · Polyunsaturated fatty acids · Tumor growth

Abstract. Diets high (17.7 cal%) and low (3.3 cal%) in linoleic acid were given to groups of Brown Norway female rats before and after inoculation of syngeneic tumor models with different characteristics, with regard to tumor spread, malignancy, immunogenicity, growth rate, rat strain, and histopathological features. Despite the differences in characteristics, in most tumor models, tumor growth was identical in both experimental groups. However, in 2 tumor models, an adrenal cortical carcinoma and a myeloid leukemia, differences in growth were noted. In rats given the diet low in linoleic acid, growth of the cortical carcinoma was significantly increased, whereas the opposite effect was seen in rats with myeloid leukemia.

Introduction

The linoleic acid content of the diet has raised much interest and has been the subject of numerous research studies in the last 2 decades. On the one hand linoleic acid in the diet is thought to be beneficial in the prevention of coronary heart disease by decreasing plasma cholesterol levels [1, 2], on the other, the incidence and growth of tumors seemed to be higher in man and animals receiving diets high in linoleic acid [2, 3]. Generally, the mechanism responsible for this higher tumor incidence is considered to be the immunosuppression which diets high in linoleic acid can exhibit [4, 5].

Studying dietary linoleic acid, with particular regard to immunosuppression and tumor promotion, it is important to consider accurately the quantity of linoleic acid in the

control diet: a diet very low or deficient in linoleic acid results in pathological conditions [6] and should never be used as a control. Also the tumor model used needs careful attention, as differences in tumor characteristics, especially with regard to immunogenicity and hormonal responsiveness, may easily lead to misinterpretation of the effects of the dietary components on tumor progression [7–9]. For these reasons a study was undertaken in which rats were given a diet high (17.7 cal%) or low but adequate (3.3 cal%) in linoleic acid, and a wide variety of tumors was inoculated, in order to investigate the tumor characteristics in relation to the alleged tumor growth-promoting effect of diets high in linoleic acid.

A comprehensive study on spontaneous tumor incidence in rats given these diets has been published elsewhere [10]. The effects of the same two diets on growth in transplantable tumor models is the subject of the present study.

Materials and Methods

Experimental Design. Groups of at least 10 animals received, after randomization, the test diets when they were 6 weeks of age. Group I was placed on a 35 cal% fat diet high in linoleic acid, group II a 35 cal% fat diet low in linoleic acid. Seven weeks after the diets were started, tumors were inoculated, after which the dietary regimen was continued. The animals were housed in macrolon cages in groups of 5, water and food was given ad lib. The animal room was air-conditioned and with a controlled 12 h light/12 h dark rhythm.

Tumors. BN472, a mammary adenocarcinoma; BN175, a soft tissue fibrosarcoma; WR1618, a skin basal cell carcinoma; BN569, a ureter squamous cell carcinoma; BN945, an adrenal cortical carcinoma; BN248, a cervix/uterus leiomyosarcoma, and BN1312, a myeloid leukemia, were used. BN tumors originated spontaneously in Brown Norway rats, the WR tumor in a Wistar/Rij rat after neutron irradiation. Apart from the myeloid leukemia, tumors were inoculated subcutaneously (pieces of approximately 5 mm^3) into the right flank of the animal. Leukemia was inoculated intravenously, 1×10^6 cells. The differences in tumor characteristics are summarized in table I. Immunogenicity of the tumors was established according to the in vivo transplantation assay as described by *Prehn and Main* [11]. Measurement of the dimensions of primary tumor growth was established weekly, using a micrometer. The mean of length and width was taken as a measure of tumor size. For tumor BN175 and BN1312 a different procedure was followed. The primary tumor of tumor BN175 was surgically removed 12 days after inoculation, 9 days later the animals were sacrificed, after which the number of metastases in the lungs was determined by counting the white nodules on the surface of the left lung. The number of metastases in the lungs was scored semiquantitatively by assigning the counted nodules into one of five categories: $\leq 10 = 1$, 11–25 = 2, 26–50 = 3, 51–100 = 4, and $> 100 = 5$. Tumor growth of tumor BN1312 was determined by weighing the spleen 16 days after i.v. inoculation of the tumor.

Dietary Regimen. Linoleic acid was given as a constituent of a semisynthetic diet. The 35 cal% fat diets contained either 17.7 (high) or 3.3 (low) cal% linoleic acid and were prepared by Unilever Research Laboratories, Vlaardingen, Holland. The composition of the diets is given in table II. Apart from the quality of dietary fats, both diets were identical in all important constituents and in caloric density. The diets were freshly prepared 3 times a week and were given to the animals every Monday, Wednesday and Friday, the uneaten food being discarded.

Statistical Evaluation. If possible, comparison of tumor growth was carried out by taking the mean values of the individual regression coefficients b ($tg\alpha$) obtained after calculating the best fitting straight line (linear regression: $y = a + bx$) from the weekly determinations of the tumor size. For tumor BN1312 the mean spleen weight at day 16 was given, while for tumor BN175 the tumor size at day 7 was given. Statistically significant differences in primary tumor growth were established using a two-tailed Student t test; the differences in number of metastases in the lungs of tumor BN175-bearing animals were tested using a Yates analysis. The differences were tested on a significance level of 5%.

Table I. Characteristics of tumor models[1]

	BN175	BN248	BN472	BN569	BN945	BN1312	WR1618
Weeks before detectable tumor growth	< 1	1	< 1	2	4–5	1	1
Growth rate[2]	4	7	6	14	10	1–2	13
Immunogenicity	–	–	–	–	+	–	+
Morphology	mes	mes	epi	epi	epi	mes	epi
Strain	BN	BN	BN	BN	BN	BN	WR
Metastasizing from site	+	–	+	–	–		–
Original anatomical site	soft tissue pancreas	cervix/ uterus	mamma	ureter	adrenal cortex	lympho-ret. system	skin
Hormone responsive[3]	+	n.e.	–	n.e.	n.e.[4]	+	n.e.

mes = Mesenchymal tumor; epi = epithelial tumor; n.e. = not established.

[1] Rats on standard rat chow (Hope Farms, AM II).

[2] Number of days between tumor size 10 mm and 20 mm diameter, except for tumor BN1312, for which the number of days between 5×10^5 and 1×10^6 tumor cells in the spleen was taken.

[3] BN175 grew more rapidly in female rats; BN472 tumor growth was identical in both sexes, whereas BN1312 grew more rapidly in ovariectomized and in adrenalectomized rats [*Kort*, unpublished data].

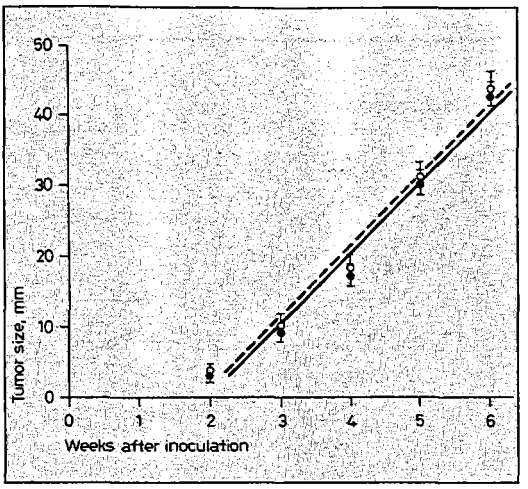
[4] Hormonal responsiveness was not tested; however, the cortical carcinoma produced steroids, as was established by atrophy of the contralateral adrenal gland [10, 23].

Results

The results of tumor growth in rats on diet high and low in linoleic acid are given in figure 1. It will be clear that apart from tumor BN945, the adrenal cortical carcinoma, no differences were observed. With this particular tumor, growth was statistically significantly inhibited in the high linoleic acid group. Furthermore, a difference was observed in rats with leukemia. In group I mean spleen weight (± SE) at day 16 was 1,196.8 ± 69.5 mg, whereas in group II, spleen weight was 946.8 ± 47.8 mg; $t(17) = 3.02$, $p < 0.01$. These results imply that in this tumor model growth was inhibited in the diet group low in linoleic acid, an effect opposite to that seen with tumor BN945-bearing animals. Finally, growth of another hormonal responsive tumor, BN175, was not significantly effected: tumor size ± SE day 7, group I, 16.1 ± 1.1 mm; group II, 14.3 ± 0.6 mm: $t(18) = 1.36$, $p < 0.19$.

The mean score (± SE) of the number of metastases in the lungs after inoculation of tumor BN175 was 3.25 (± 0.5) and 2.70 (±0.3) in groups I and II, respectively. The difference was not statistically significant (Yates t score = 0.92, $p < 0.65$). The median of the number of metastases in both experimental groups was identical: 39.5.

Tumor BN472 grew substantially less rapidly on the semisynthetic diets as used in the present study in comparison with the growth observed in BN rats on commercial diet, in which invariably, using the same experimental design, metastases could be established. Probably as a result of this, at the time of sacrifice, no tumor spread to regional lymph nodes could be found in either dietary group.



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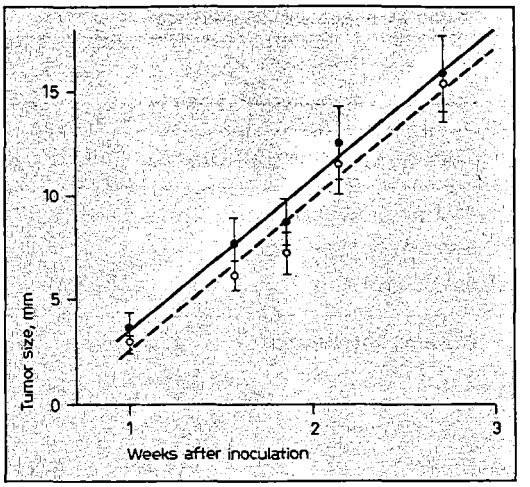

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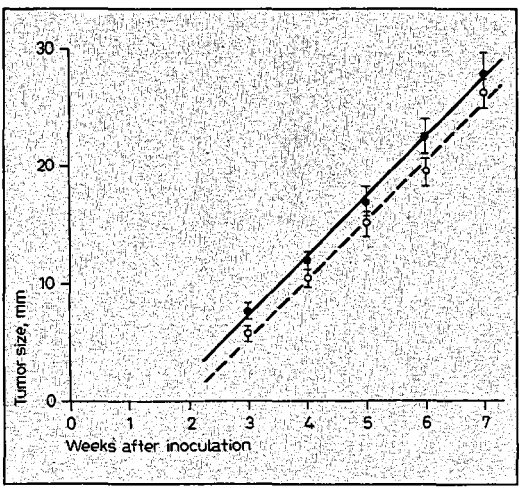

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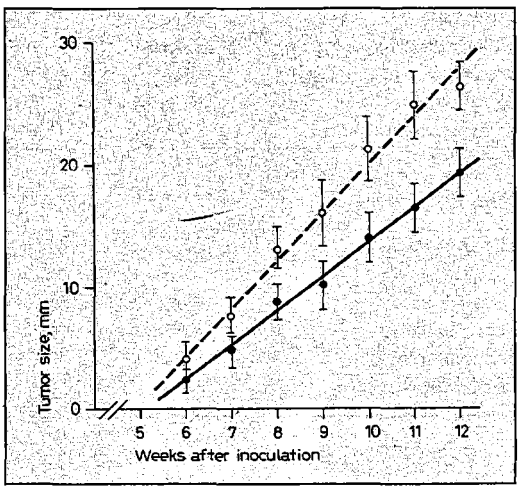

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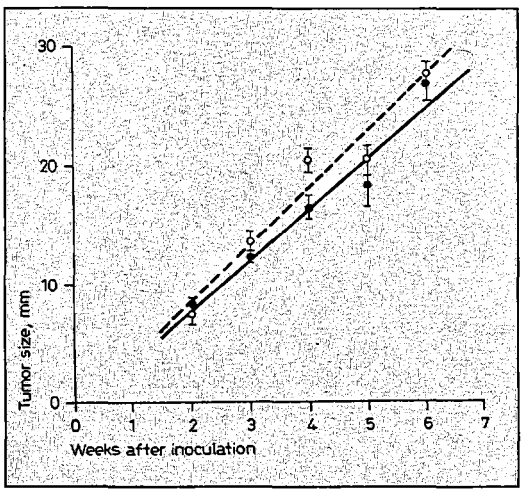

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Fig. 1. Growth of transplantable tumors on diet high and low in linoleic acid. ●——● = Group I, 17.7 cal% linoleic acid; ○- - -○ = group II, 3.3 cal% linoleic acid. The vertical bars represent the SEM.

a Tumor BN248, cervix/uterus leiomyosarcoma: tgα (± SE) linoleic acid high diet, 10.28 ± 0.39; tgα (± SE) linoleic acid low diet, 10.22 ± 0.29; $t(18) = 0.15$, $p < 0.88$, not significant.

b Tumor BN472, mamma adenocarcinoma: tgα (± SE) linoleic acid high diet, 7.96 ± 0.60; tgα (± SE) linoleic acid low diet, 8.20 ± 0.74; $t(25) = 0.28$, $p < 0.78$, not significant.

c Tumor BN569, ureter squamous cell carcinoma: tgα (± SE) linoleic acid high diet, 5.06 ± 0.35; tgα (± SE) linoleic acid low diet, 5.02 ± 0.29; $t(18) = 0.87$, $p < 0.39$, not significant.

Table II. Composition of the diets

	Group I	Group II
Composition of the diets as a percentage of the energy		
Protein	23	23
Fat	35	35
Of which linoleic acid	17.3	3.3
Carbohydrates	42	42
Distribution (1,000 kcal)		
Casein	64.111	64.111
Palm oil	–	37.634
Corn oil	28.0	–
Sunflower oil	9.634	–
Starch	120.0	120.0
Minerals (Cas. '73)[1]	5.354	5.354
Vitamins (Vit. '70)[2]	1.0	1.0
Cellulose	15.0	15.0
Total	243.099	243.099
Composition of the fatty acids (percentage)[3]		
Palmitic acid	11.1	45.8
Stearic acid	3.3	4.3
Oleic acid	32.4	38.2
Linoleic acid	50.7	9.5
Linolenic acid	0.9	0.2
Arachidonic acid	1.6	2.0

[1] Cas. '73: In mg/1,000 kcal, potassium chloride, 350; sec. magnesium phosphate, 956; prim. potassium phosphate, 475; potassium bicarbonate, 719; calcium carbonate, 2,014; trisodium citrate 2 aq., 711; manganese sulfate, 67.8; ferric (III) citrate, 43.9; copper citrate, 4.7; zinc citrate, 12.5; potassium iodate, 0.07.

[2] Vit. '70: In mg/1,000 kcal, choline (50%), 500; vitamin E (250 IU/g), 80; calcium silicate, 50; myo-inositol, 25; vitamin B_{12} (1,000 mg/kg), 5; vitamin A (325 IU/mg), 7.7; nicotinic acid, 5; (calcium)-pantothenic acid, 5; riboflavin, 1.5; thiamin monocitrate, 1.5; vitamin D (80 IU/mg), 3.125; vitamin K_3 (22.7%), 1; pyridoxine, 0.5; folic acid, 0.25; biotin, 0.05; sucrose, 314.375.

[3] Established by means of gas chromatography.

Discussion

In an earlier study, we reported that a semisynthetic diet with 35 cal% fat, consisting predominantly of polyunsaturated fatty acids (PUFA), prolonged kidney allograft survival in rats [12]; however, a diet containing the same amount of fat in which sunflower oil was replaced by palm oil was almost as effective. The finding that diets high in fat exerted an immunosuppressive effect was observed in a number of other studies as well [4, 5, 13, 14]. However, the PUFA level of the diet was claimed to be responsible for the immunosuppressive effect of high fat diets [13, 15]. Conversely, immuno-potentiating effects were described when diets low or deficient in linoleic acid were given [14]. However, in parallel studies by us, using the same amounts of linoleic acid as were given in the present study, no such difference between high and low PUFA diets on cellular immune response could be established [16]. But when the immune capacity was related to that of rats on standard rat chow, which is relatively low in fat but also differs with regard to other dietary constituents, a decrease in immune response was observed [16]. This discrepancy between our results and those of others referred to above might be traced back to the chosen levels of PUFA in the diets. Especially with regard to the low PUFA diet, one has to be careful to avoid linoleic acid deficiency, as this certainly will give pathological conditions, such as a suppression of blastogenesis [6]. Our postulation is supported by the finding of *Hillyard and Abraham* [17], who in a study on the growth-promoting effect of dietary PUFA on transplantable mouse adenocarcinoma gave increasing amounts of linoleic acid and found that addition of as little as

0.1% of linoleic acid to the fat-free diet was enough to abolish the tumor growth-inhibiting effect of such a diet.

In addition to the direct consequences of the immuno-inhibiting effects of high fat diets on, for instance, virus or bacterial susceptibility [5], there is substantial evidence that high fat diets also enhance tumor growth [5, 18], although, recently it has been shown that the overall energy intake is of primary importance to the observed increase of tumor incidence, independent of the fat content or distribution of the fatty acids in the diets [19]. Also the role of linoleic acid, which initially was considered as the most important constituent of dietary fat promoting tumorigenesis, is still subject to much debate [20, 21].

In our study on transplantable tumor growth, enhanced tumor growth in a high PUFA diet was observed only in the BN1312, myeloid leukemia, tumor growth. As for all other tumor models, a high amount of PUFA in the diet did not contribute to enhancement of tumor growth. On the contrary, in tumor BN945, the adrenal cortical carcinoma, a substantial increase in tumor growth is observed in the rats given the diet low in linoleic acid. Furthermore, the results gathered from a longevity study, in which rats were given the experimental diets, did not show important differences in total tumor incidence nor in tumor incidence of any particular tumor site [10]. In the group fed a diet high on linoleic acid, 149 tumors were found in 71 tumor-bearing animals (out of 100 animals); and in the group fed a diet low in linoleic acid, 123 in 75 animals (out of 100 animals). This difference was not statistically significant in a Peto analysis, which is chi-square analysis adapted for evaluation of cumulative incidence data. It was interesting, however, that in this study differences in adrenal gland tumors and lymphoreticular tumors were observed. Both tumor incidences were significantly lower in the groups with the high intake of linoleic acid. As both tumors are known to be responsive to the hormonal environment, certainly with regard to corticosteroids (table I) [22, 23], it is possible that this specific characteristic contributed to the observed phenomenon. Support for such a possible involvement of hormones is found in other studies from our group in which, using the same diets, significantly different levels of insulin and corticosterone were established [16, 24]. Also in other tumor models, dietary-induced changes in hormone levels were held responsible for the observed differences in tumor growth [25]. In this respect the hormone prolactin has been mentioned [26], although recent literature discounts a possible involvement of prolactin in tumor growth [27]. However, as we do not have any information concerning tumor growth responsiveness of myeloid leukemia and cortical carcinomas to this hormone or to a number of other hormones, a contribution via such a mechanism cannot be excluded.

Also in the longevity study [10], one could conclude from the high incidence of death due to infectious diseases that the immune capacity of all animals on the 35 cal% fat-containing semisynthetic diet, regardless of its linoleic acid content, was depressed compared with animals on standard Hope Farms

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d Tumor BN945, adrenal gland cortical carcinoma: tgα ($\pm$ SE) linoleic acid high diet, 2.84 $\pm$ 0.25; tgα ($\pm$ SE) linoleic acid low diet, 3.80 $\pm$ 0.25; $t(17) = 2.73$, $p < 0.01$, significant.

e Tumor WR1618, skin basal cell carcinoma: tgα ($\pm$ SE) linoleic acid high diet, 4.78 $\pm$ 0.37; tgα ($\pm$ SE) linoleic acid low diet, 5.01 $\pm$ 0.39; $t(16) = 0.42$, $p < 0.68$, not significant.

rat chow. However, this temporarily or more permanently induced immunosuppression did not result in a substantially higher tumor incidence, when compared with other studies [23]. For comparison, in this longevity study a total of 410 tumors were found in 208 (88%) of total) tumor-bearing animals in the BN female rat used [23].

Although it seems obvious from studies performed by others as well as by our group that certain diets may exhibit immuno-modulating properties, a relation beween this effect and differences in growth of nonimmunogenic tumors seems farfetched [28]. Except for the special cases of lymphoreticular or viral induced tumor growth there is not a greatly increased tumor incidence either in patients immunosuppressed after transplantation or in immunodeficient animals [5]. Although so far the immunosurveillance theory [29] cannot be excluded completely, other explanations for differences in tumor growth seem to get more support. Apart from the earlier discussed theories with regard to the contribution of hormonal alterations as being tumor mediating, a number of other possibilities are open for discussion. Immunosuppression as well as hormonal activity play a more central role by modifying host resistance, but increasing amounts of PUFA in the diet also modify the fatty acid composition of the cell membrane and, by doing so, change the cell kinetics, resulting in e.g. an alteration of cholesterol (decreased [30]) or prostaglandin synthesis (increased [31]). With regard to the latter, prostaglandin synthesis has a number of interesting aspects: malignancy and even tumor spread are claimed to be correlated with increased tumor cell prostaglandin activity [32, 33].

From the results obtained we conclude that there is no reason to presume that, with the amounts of PUFA that were given in our studies, a high linoleic acid content of the diet is related to enhanced tumor growth by means of inducing immunosuppression. The reason for the differences in tumor growth, as found in the present study, remains unclear, but bearing in mind the specific tumor characteristics of the 2 tumors apparently sensitive to the PUFA content of the diet (the myeloid leukemia and the cortical carcinoma), it seems that hormonal responsiveness with regard to e.g. corticosteroids and prolactin might be involved, both in a negative or a positive way.

This study proved once again that dietary factors can react differently in different tumor systems. This leads us to another conclusion: that, whenever dietary effects on tumor growth are judged, this should preferably be done on the basis of the results of more than 1 tumor model, as the specific characteristics of a given tumor model are apparently of major importance to the results obtained.

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Publication VII

OMEGA-3 FATTY ACIDS INHIBIT THE GROWTH OF A TRANSPLANTABLE MAMMARY ADENOCARCINOMA

This article has been submitted for publication.

OMEGA-3 FATTY ACIDS INHIBIT THE GROWTH OF A TRANSPLANTABLE RAT MAMMARY ADENOCARCINOMA

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Abstract

Rats fed diets containing different amounts of polyunsaturated fatty acids either of the omega-3 or omega-6 type, received an implant of a syngeneic mammary adenocarcinoma. When the diameter of the tumors reached 20 mms, they were surgically removed; 2 weeks thereafter the animals were sacrificed and lung metastases were counted. Cellular immune response was determined before tumor inoculation; certain prostaglandin values in plasma, and platelet aggregation were measured before and after tumor inoculation.

Plasma PGE_2 and TXB_2 values were significantly decreased in those rats fed a diet containing Menhaden oil. 6-keto-$PGF_{1\alpha}$, cellular immune response and platelet function were not significantly different in either one of the diet groups.

Tumor growth in the groups of rats receiving the omega-3 fatty acids in their diet was significantly inhibited in comparison with the rats receiving the omega-6 fatty acids. However, the number of metastases was not significantly altered.

Introduction

Epidemiologically, there is "suggestive evidence" for a causal relationship between dietary fat intake and some cancers [1-3]. In animal studies a positive correlation between dietary fat content and mammary tumor initiation or promotion could be demonstrated [4-6]. In particular diets rich in linoleic acid, were claimed to have mammary tumor promoting capacities in comparison with diets which were low or deficient in linoleic acid [5,6]. In this respect, omega-3 type of polyunsaturated fatty acids recently received a lot of interest too. These fatty acids, mainly present in fish oil, are consumed in relatively large quantities by Eskimos and to a lesser extent by groups of Japanese fishermen [7,8]. Epidemiological data showed that the low prevalence of colon and breast cancer among Greenland Eskimos and Japanese could be correlated with the quality of their low fat intake [9-11].

An other important finding in Greenland Eskimos, which has been associated to their dietary habits, is the very low incidence of ischemic heart disease [12,13]. This prevention of coronary heart disease is considered to be the result of changes in blood platelet function, induced by the fatty acids EPA and DHA, which are prominent polyunsaturated fatty acids present in some marine lipids [13,14]. The anti aggregatory activity of these fatty acids is either attributed to to the formation of Prostaglandin-I_3 [12,15] or to a competitive inhibition by EPA and DHA of the biosynthesis of TXA_2 from arachidonic acid, resulting in a reduction of its pro-aggregatory activity on platelets [16]. The eventual effect of both theories is the same: they lead to decreased thrombosis tendency and prolonged bleeding time.

Prostaglandins may also play an important role in tumor initiation, promotion and spread [17-19]; one might therefore presume that the observed low incidence of mammary cancers amoung the Japanese is related to changes in PG metabolism induced by the specific dietary lipid composition of their diet. However, one should not forget that the incidence of e.g. stomach cancer amoung Japanese is very high, which is the reason that the overall cancer frequency is similar to that of Caucasians.

In the present study the effect of 4 different diets was investigated. Two of the diets contained, next to 3 en% linoleic acid, different amounts of fish oil, the others did not contain EPA or DHA, but 3 and 10 en% of linoleic acid, respectively. The diets were given to rats, before and after inoculation of a

transplantable mammary adenocarcinoma, to study the influence of the diets on tumor growth and the formation of metastases. Some stable plasma-PG metabolites and platelet aggregation were measured in order to correlate possible alterations in PG synthesis with differences in tumor growth. Cellular immune capacity was tested as well, as the diets under study may exhibit changes in immune response and by this affect tumor growth.

Materials and methods

Experimental design.-A total number of 100 BN/Bi inbred female rats (Zentral Institut für Versuchstierzucht, Hannover, Germany) entered the study at the age of 3 weeks (immediately after weening). At arrival the animals were randomly selected, divided into 4 groups and put on one of 4 experimental diets. The rats were housed 5 per Makrolon cage on wood chips bedding, receiving food and water ad libitum. The animal room was air-conditioned and had a controlled night-dark rhythm.

Every week the animals were weighed, and twice a week the average food consumption was measured. At the age of 6, 9, 12, 15 and 17 weeks of age, in each experimental group, 2 animals were sacrificed for plasma PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 determinations and ADP-induced platelet aggregation. Twice, at the age of 9 and 12 weeks, 2 rats in each group were killed for testing cellular immune response, by determining Con A-, and PHA-induced mitogen stimulation, and NK cell activity. Tumor inoculation of tumor BN472, a mammary adenocarcinoma, was performed when the animals were 12 weeks of age, i.e. 9 weeks on diet. Of the remaining 21 animals in each group, 15 rats received viable tumor tissue, whereas, 6 rats received γ-irradiation-killed tumor tissue. Tumor size was established by palpation every other day in the period between tumor inoculation and removal of the primary tumor, which was carried out when the tumor reached a diameter of 20 mm. The experiment was terminated exactly 2 weeks after primary tumor removal, at which time the remaining animals (11 rats with tumors, and 2 control animals, which had received γ-irradiated tumor tissue) were sacrificed in order to determine the number of metastases in lungs and regional lymph nodes. In 2 of the tumor bearing rats and in the 2 control rats out of each dietary group, plasma PG metabolites and ADP-induced platelet aggregation were measured as well.

Diets.-The four experimental diets were identical, each containing 30 en% of fat, but were differently composed with

Table I

CHARACTERISTICS OF THE STUDY DIETS

Composition of the experimental diets (g/1,000 Kcal)

Diet Compound	diet groups A-D
Casein	62.000
Minerals (Cas. '85)[a]	8.458
Vitamins (Vit. '70)[b]	1.000
Cellulose	15.000
Starch	134.286
Fatty acids	32.3

Sources of fatty acids (g/1,000 Kcal)

	diet group A	B	C	D
cacao butter	24.946	13.656	–	7.204
sunflower oil	2.581	14.194	3.441	1.613
olive oil	4.839	4.409	0.108	13.118
linseed oil	–	–	3.226	–
Menhaden oil	–	–	25.376	10.323
total	32.366	32.259	32.151	32.258

Composition of the fatty acids (en %)[c]

	diet group A	B	C	D
saturated fatty acids	15	10	10	10
monoenoic fatty acids	12	10	10	15
linoleic acid	3	10	3	3
EPA and DHA	0	0	7	2
total	30	30	30	30
P/S ratio[d]	0.2	1.0	1.0	0.5

a) In mg/1,000 Kcal: potassium chloride, 350; secondary magnesium phosphate, 956; primary potassium phosphate, 475; potassium bicarbonate, 719; calcium carbonate, 2014; trisodium citrate $2H_2O$, 711; manganese sulphate, 51.4; ferric citrate, 43.9; copper citrate, 4.7; zinc citrate, 12.5; potassium iodate, 0.07; tri-sodium dicitrate, 1630; magnesium acetate, 1490.

b) In mg/1,000 Kcal: choline (50%), 500; vitamin E (500 IU/g), 40; calcium silicate, 50; myo-inositol, 25; vitamin B_{12} (1,000 mg/kg), 5; vitamin A (325 IU/mg), 7.7; nicotinic acid, 5; (calcium)-panthothenic acid, 5; riboflavin, 1.5; thiamine mononitrate, 1.5; vitamin D (80 IU/mg); 3.125; vitamin K_3, (22.7%), 1; pyridoxine, 0.5; folic acid, 0.25; biotin, 0.05; sucrose, 354.375.

c) Established by gas chromatography.

d) P/S ratio = ratio between polyunsaturated fatty acids and saturated fatty acids.

regard to their fatty acid content (Table 1). Diets A and B contained 3, respectively 10 en% of linoleic acid; diets C and D contained 3 en% of linoleic acid (like diet A), but also 7 and 2 en% of EPA and DHA, respectively. The diets were prepared at Unilever Research Laboratories, Vlaardingen, The Netherlands. The diets were freshly prepared weekly. To prevent peroxide formation, the diets were stored *in vacuo* in sealed plastic bags. The animals received fresh food every Monday, Wednesday and Friday; at the same time the uneaten food was discarded.

Measurement of food consumption.-Every Monday and Wednesday a measured quantity of fresh food was given to the animals. Two days later food remnants were collected and weighed to determine food consumption per cage. By dividing this number by 10 (number of days x number of rats per cage) the approximated food consumption per rat per day was obtained.

Tumor inoculation.-The tumor used in this study, originally classified as a malignant mammary adenocarcinoma (BN472), is one of the transplantable tumors routinely used in our studies on tumor growth and metastases [20]. The tumor metastatises from site to regional lymph nodes and lungs. Under light ether anesthesia a small incision was made in the skin of the right flank, and a small piece of tumor tissue (5-10 mm^3), obtained from a syngeneic rat, was implanted s.c. The wound was closed with an autoclip. From day 7 onwards, at which day the clip was removed, the tumor dimensions were measured every other day using calipers, taking (length + width)/2 as the "tumor diameter". When the tumor diameter reached 20 mm, the tumor was surgically removed, again under ether anesthesia. The operation was performed under clean but non sterile conditions. The tumor mass was removed as much as possible, and in an attempt to kill the remaining tumor cells, the wound was moistened with a 4% formaldehyde solution. Bleeding was controlled by bipolar coagulation. The wound was closed with autoclips. The procedure for those rats receiving γ-irradiated tumor tissue instead of viable tumor, was identical to the procedure described above, with the exception that the pieces of tumor tissue were γ-irradiated with 15,000 Rads in 2 hours.

Two weeks after removal of the primary tumor the animals were sacrificed and autopsied. Regional lymph nodes (axial, inguinal and para-aortal) were examined (macroscopically) for signs of tumor metastasis and the lungs were fixated in Bouin's fixative for microscopical examination of metastasis. This latter was performed by counting, at a magnification of 400x, the number of metastases in a 3μm thick section of the left lung (longitidunally). Lung metastases were scored semiquantitatively

by the score: 0=0; 1-5=1; 6-10=2; 11-20=3; 21-30=4; >30=5.

Determination of cellular immune response.-According to the schema as been given in ***experimental design*** animals were sacrificed for immune response measurements. To prepare the spleen cells necessary for the assays, spleens were chopped and passed through a 100 μm gauze sieve. For mitogen stimulation, cells were washed three times and the cell concentration adjusted to 7.5×10^6/ml in RPMI, 10% foetal calf serum. For the NK cell assay lymphocytes were prepared by gradient centrifugation [21]. Lymphocytes were collected and depleted of adherent cells by 1 hour incubation at 37°C in a tissue culture flask. Non adherent cells were resuspended at a concentration of 20.10^6/ml in RPMI (Gibco, UK) to which also 10% foetal calf serum was added.

For mitogen stimulation 20 μl of a 50 μg/ml Con A solution (Pharmacia, Holland) or 20 μl of a 25 μg/ml PHA solution (Wellcome, UK) was added to 1.5×10^5 lymphocytes, which were cultured in Microtitre culture plates (Greiner, W. Germany), at 37°C in a humidified atmosphere of 5% CO_2. Harvesting was performed on day 3 of culture. At 6 hours before harvesting, 0.8 μCi of methyl-[^{3}H]-thymidine (specific activity, 2 Ci/mmol; Amersham TRA 310) was added to each well. Con A and PHA stimulation was expressed as counts/min/culture (containing 0.15×10^6 lymphocytes).

The NK cell assay was performed as described before [22], with a few small modifications, using YAC-1 lymphoma cells as a target. Shortly: effector cells were incubated for 4 hours with [^{51}Cr] labelled YAC cells with an effector to target cell ratio of 200:1. Specific lysis was calculated according to the formula:

$$\% \text{ specific lysis} = \frac{\text{mean experimental lysis} - \text{mean spontaneous lysis}}{\text{mean maximum release} - \text{mean spontaneous release}} \times 100$$

In vitro platelet aggregation assay.-Under ether anesthesia the animals received a intravenous dose of 50 IU heparin, after which the abdomen was opened, the abdominal aorta was dissected at its bifurcation, and blood was collected by aortic puncture. The rats were then sacrificed. 5 ml blood samples were equally divided into two plastic test tubes, one containing an extra amount of 10 μl of Heparin (5000 IU/ml), which was for the platelet aggregation determination. Into the other test tube the blood was put for the measurement of PG-endproducts. After a rest period of 30 minutes (to eliminate possible effects of endogenous TXA_2 and PGI_2), the blood platelets were counted in a TOA Platelet Counter PL100. Platelet function was measured by using a Chronolog-Whole Blood Aggrometer (Chronolog Corp., UK).

In siliconized glass cuvettes, which were kept at 37°C, under constant stirring 0.5 ml of saline was added to 0.5 ml of whole blood. To induce platelet aggregation 10 μM ADP was added to the sample. The changes in electrical resistance induced by the platelet aggregation were recorded during 10 minutes on an Olivetti M24 PC, which calculated the degree of maximum aggregation, which was taken as a measure of platelet function.

Determination of prostaglandins.-6-keto-$PGF_{1\alpha}$, TXB_2 (the stable degradation products of PGI_2 and TXA_2, respectively), and PGE_2, were determined, in the same blood sample as used for platelet aggregation, by means of a RIA, with the use of rabbit antisera (anti-6-keto-$PGF_{1\alpha}$, Seragen, Boston, USA; anti-TXB_2 and -PGE_2, Institut Pasteur, Paris, France; cross reactivity of these anti-sera with each other was <.1% at 50% B/Bo). To the 2.5 ml heparinized blood sample, 250 μl 2.8% of EDTA and 10 μl of a 0.3% Indomethacin solution (in ethanol abs.) was added immediately after bleeding the animals. Within the next half hour, the samples were centrifuged and plasma was stored at -70°C until they were assayed.

Statistics.-A two way variance analysis was used to test the differences in cellular immune response, platelet aggregation and PG determinations. A one way variance analysis was used to compare differences in tumor size and metatases. In case of significant differences (i.e., an F value indicating a probablity <.05), the Newman Keuls test was used to find which pair(s) of means were statistically significantly different [23]. A Yates analysis [24] was used to test the differences in the score of metastasis.

Results

Animals and diets

The animals accepted the experimental diets well, and no significant differences in food consumption were observed (Table 2). There were also no statistical significant differences in growth rate (Figure 1). Peroxidation was measured in food samples, which were left at room temperature for 3 days, which was the maximum of exposure to air and room temperature. Values found were: 4.2, 12.1, 32.2, 15.2 milli equivalent O_2/kg fat, for diet groups A, B, C, and D, respectively. These low values indicate that peroxidation was minimal.

Table 2. – Food consumption/rat/day (grams)[ab]

wks of age	wks after inocu-lation	diet group A	B	C	D
4	– 8	6.3 (.15)	6.2 (.18)	6.1 (.20)	6.2 (.07)
6	– 6	6.2 (.16)	6.2 (.12)	6.3 (.10)	6.3 (.12)
9	– 3	7.0 (.10)	6.8 (.20)	6.8 (.21)	6.8 (.13)
12	0	7.6 (.17)	7.0 (.12)	6.7 (.25)	6.7 (.20)
14	2	7.6 (.23)	7.2 (.11)	7.1 (.17)	7.1 (.18)

a) See for composition experimental diets, Table 1. Values given in parenthesis, SE.
b) No statistical significant differences in food consumption in any of the dietary groups.

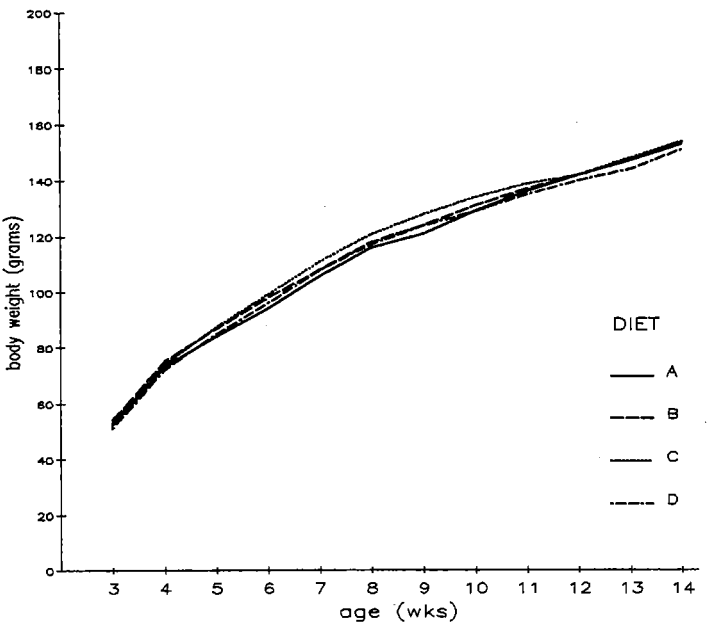

Figure 1. – Body weight gain of rats between week 3 and week 14 of age. No significant differences between diet groups A-D.

Cellular immune response

In Table 3, Con A, PHA and NK cell activities are given. Especially the results of the PHA test determinations at wk 12 of age, coinciding with the inoculation procedure, showed in all dietary groups significantly decreased values when compared with the values obtained 3 wks previously. The similarity of this decrease in PHA activity was striking: the percentile decrease {100% -(12 wks values:9 wks values)x100} was 29.9, 31.6, 30.5, 29.1% in groups A-D, respectively.

Especially at wk 9, but also, to a somewhat lesser degree, at wk 12, total white blood cell counts in the spleens of the rats in group C were strikingly higher compared with the rats in other groups. However, also due to the limited number of observations, this difference was not statistically significant.

In the NK cell activity assay (Table 3) no statistically significant differences were observed, neither in any of the dietary groups nor between wk 9 and wk 12 values.

Table 3. - Cellular immune response[a]

wks of age	diet group A	B	C	D
Con A (counts/min × 10^3 in .15 × 10^6 spleen cells)				
9	175,363	156,998	185,880	175,433
	23,330	8,956	7,653	34,883
12	185,579	157,115	152,654	112,262
	11,223	18,540	26,845	44,301
PHA (count/min × 10^3 in 1.5 × 10^5 spleen cells)				
9	90,886	97,372	91,668	93,637
	2,620	6,104	470	5,918
12[b]	63,748	66,628	63,747	66,410
	1,824	2,440	10,075	5,461
NK cell activity (% specific lysis)				
9	18.6	17.8	17.4	22.4
	(8.5)	(3.6)	(4.8)	(1.3)
12	16.7	19.6	13.6	13.2
	(4.0)	(9.6)	(3.1)	(.2)
Total white blood cell count/spleen (× 10^7)				
9	34.6	32.2	47.0	35.2
	(4.6)	(4.0)	(2.0)	(5.6)
12	34.5	30.9	37.2	32.0
	(4.9)	(13.2)	(1.4)	(5.6)

a) See for composition experimental diets, Table 1. Values given in parenthesis, SEM
b) Values statistically significant decreased, compared with values week 9.

Tumor growth and metastasis

From the total number of 52 animals receiving a piece of viable tumor tissue, 2 tumor inoculae did not grow: 1 in group C, the other in group D, these animals were not included in any further evaluation. The 24 animals receiving pieces of irradiated tumor did not show any signs of tumor growth, nor of any inflammatory reaction. In Table 4 values of "tumor growth rate" (mean number of days to reach a tumor diameter of 20 mm), and the metastatic score are given. Rats in group C as well as in group D showed statistically significantly inhibited growth of their tumors ($p<.05$), as it took these rats 5-6 days more to reach a tumor diameter of 20 mm.

Neither the number of tumor metastases in the lungs were statistically significantly different, nor was tumor spread to regional lymph nodes (Table 4).

Table 4. - Mean tumor growth rate and score of metastasis[a]

	diet group			
	A	B	C	D
Mean tumor growth rate[b]	19.2 (.9)	20.5 (1.5)	26.8[c] (2.9)	25.0[c] (1.7)
Tumor metastasis score[d]				
0	1	2	2	3
1-5	11	6	7	5
6-10	-	1	1	3
11-20	1	1	1	1
21-30	-	1	1	-
<30	-	2	-	-
No. of rats with pos. ly. nodes/total No. of rats	1/13	2/13	0/12	0/12

a) See for composition experimental diets, Table 1. Values given in parenthesis, SEM.
b) Mean tumor growth rate = Mean number of days before the tumor reached a diameter of 20 mm.
c) Differences statistically significant vs diet groups A and B, other differences are not statistically significant.
d) Differences in tumor metastasis score are not statistically significant.

Plasma PG-determinations

Plasma PG values are given in Table 5. In these tables, values of plasma PG determinations of animals before tumor inoculation (values at wk 6 and wk 9 of age), 2 days after inoculation of the tumor (wk 12), when tumor size was 20 mms (wk 15), and at termination (wk 17), are given. The last 3 determinations were carried out in animals either receiving a piece of viable tumor tissue, or a piece of γ-irradiated (killed)

Table 5. – Plasma Prostaglandin values[a]

6-keto-PGF $_{1\alpha}$ (pg/ml plasma)

wks of age	wks after inoculation	diet group A	γA	B	γB	C	γC	D	γD
6	−6		71 (17)		117 (30)		61 (1)		313 (191)
9	−3		293 (59)		222 (14)		141 (2)		233 (91)
12	0	136 (20)	331 (130)	191 (13)	171 (23)	153 (4)	145 (5)	242 (106)	248 (114)
15[b]	3	96 (14)	94 (31)	154 (10)	113 (6)	115 (16)	104 (9)	131 (12)	97 (20)
17[b]	5	172 (9)	162 (43)	105 (4)	111 (0)	54 (14)	33 (13)	100 (62)	42 (11)

PGE_2 (pg/ml plasma)

wks of age	wks after inoculation	diet group A	γA	B	γB	C[c]	γC[c]	D[c]	γD[c]
6[d]	−6		28 (7)		34 (3)		31 (9)		13 (3)
9	−3		8 (6)		7 (2)		4 (3)		3 (2)
12	0	5 (4)	8 (3)	9 (1)	10 (2)	6 (0)	5 (4)	6 (2)	5 (1)
15	3	9 (1)	8 (3)	3 (2)	9 (4)	2 (1)	2 (1)	5 (1)	1 (0)
17	5	15 (7)	13 (3)	24 (17)	12 (3)	3 (1)	3 (2)	3 (2)	2 (1)

TXB_2 (pg/ml plasma)

wks of age	wks after inoculation	diet group A	γA	B	γB	C	γC	D	γD
6	−6		34 (3)		51 (19)		1 (0)		8 (7)
9	−3		1 (0)		1 (0)		1 (0)		1 (0)
12	0	9 (8)	3 (3)	41 (3)	33 (4)	23 (10)	1 (0)	1 (0)	1 (0)
15[e]	3	138 (91)	43 (1)	64 (39)	62 (23)	126 (25)	28 (18)	45 (43)	7 (6)
17	5	49 (24)	14 (11)	33 (21)	14 (6)	1 (0)	1 (0)	1 (0)	1 (0)

a) See for composition experimental diets, Table 1. Values given in parenthesis, SEM; Groups γA-γD received γ-irradiated tumor tissue instead of viable tumor tissue.
b) Values obtained at week 3 and 5 after tumor-, or sham- inoculation are statistically significant ($p < .05$) decreased compared with pre-inoculation values.
c) Rats in diet groups C and D show statistically significant ($p < .05$) inhibited values compared with rats in group A and B.
d) Values determined at week 6 of age are statistically significant elevated, compared with values

tumor. These latter values are thus sham control values, exclusively showing dietary influences, and those effects due to the sham tumor inoculation procedure.

6-keto-$PGF_{1\alpha}$ values were not influenced by the diets, however, after tumor inoculation, in all diet groups, a decrease was established. Determinations 3 and 5 wks after tumor inoculation were statistically significanly decreased compared with previous values. However, this decrease was not found exclusively in the animals in which viable tumor tissue was inoculated but was also seen in those animals with γ-irradiated tumor tissue. This shows that not the tumor as such, but the procedure of tumor inoculation results in the observed decrease of 6-keto-$PGF_{1\alpha}$.

Also PGE_2 values showed significant differences; these differences were two sided, i.e., not only differences were noted, which could be attributed to the experimental diets, also an effect independent of the diets was observed. In comparing "column-wise", which show differences, which are the result of dietary influences, in diet groups C and D, PGE_2 synthesis was significantly inhibited, when compared with groups A and B ($p<.05$). In comparing "row-wise", the first determination of PG's showed (when the animals were 6 weeks of age) significantly lower values, when compared with next determinations ($p<.01$).

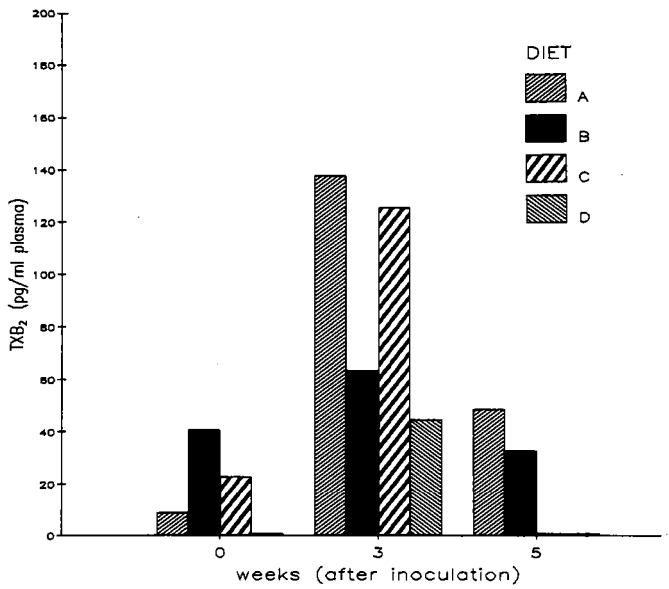

Figure 2. – Plasma TXB_2 values 2 days (week 0), 3 and 5 weeks after tumor inoculation. At week 3, TXB_2 values are statistically significant increased ($p < .05$).

Finally, TXB_2 values showed statistically significant differences as well, more or less comparable with the previously stated PGE_2 determinations. In diet groups C and D, TXB_2 synthesis was also significantly inhibited compared with groups A and B ($p<.05$). Furthermore, a strong increase in TXB_2 values was noted at wk 3 after inoculation, coinciding with the period that the rats bore tumors of 20 mms. This difference was especially prominent is rats, in which vitale tumor tissue was inoculated, however, in sham operated control rats an increase in TXB_2 values was seen as well. At wk 5 after inoculation no effect of the tumor could be observed any more, neither in tumor bearing animals, nor in sham operated animals. Figure 2 specifically shows the aspects of tumor growth on TXB_2 values in the different diet groups.

Platelet aggregation

Platelet aggregation expressed as maximum aggregation is given in Table 6. The values, representing the maximum aggregation recorded by ADP-induced aggregation in whole blood, show a variable pattern. The large variation was not due to changes in the number of platelets, as these were not influenced by diet or tumor progression. Before tumor inoculation, values ($x10^6$/ml blood) were, mean±SE (n=4): 684±56.0; 701±39.1; 631±55.8; 699±86.1, and after inoculation, mean±SE (n=6): 662±53.7; 631±35.1; 678±36.7; 607±60.3. The differences were not statistically significant.

Table 6. – Maximum ADP-aggregation (values in 'Ω')[a,b]

wks of age	wks after inoculation	diet group							
		A	γA	B	γB	C	γC	D	γD
6	−6	22.0 (7.1)		30.0 (1.3)		16.0 (.3)		21.8 (1.0)	
9	−3	17.2 (7.2)		17.7 (3.2)		29.3 (2.4)		18.8 (.4)	
12	0	27.1 (1.1)	24.1 (4.5)	23.9 (5.3)	18.9 (.8)	22.4 (.4)	23.0 (.6)	20.5 (3.8)	20.1 (3.4)
15	3	11.6 (3.5)	17.3 (1.4)	25.4 (1.5)	18.6 (1.2)	15.2 (2.9)	17.8 (.3)	17.3 (5.2)	21.1 (1.0)
17	5	31.3 (3.6)	23.9 (2.4)	23.2 (1.0)	26.1 (4.2)	22.0 (1.3)	17.8 (.3)	20.0 (2.5)	14.7 (1.9)

a) See for composition experimental diets, Table 1. Values given in parenthesis, SEM; Groups γA-γD received γ-irradiated tumor tissue instead of vitale tumor tissue.
b) No statistical significant differences.

The effect of tumor growth on platelet function could be demonstrated by some small differences in the ADP-aggregation. 3 weeks after tumor inoculation, in all dietary groups exept for group B, a non statistically significant decrease in platelet function was observed. 2 wks later, at termination, this temporarely decrease could not be established anymore. No (statistically significant) differences between the 4 diet groups were observed.

Discussion

The possible association between the specific dietary habits of Greenland Eskimos and ischemic heart disease has been known for some time [12,13], but a possible association with their diet and carcinogenesis has been investigated only more recently [9,25]. Some investigators [26-28] studied in experimental rat tumor models, the effects of diets containing certain amounts of omega-3 polyunsaturated fatty acids on tumor progression. Their first results confirm the validity of the hypothesis that EPA and DHA in the diet might reduce mammary tumor growth [26-28].

The most important features of feeding omega-3 fatty acids are the change in cell membrane composition and the alterations in PG-synthesis these fatty acids exhibit [29,30]. These changes are responsible for the observed prolonged bleeding time in humans [13], and possibly by this effect, also for the favorable effect on coronary heart disease. The possible significance of PG synthesis with regard to tumor initiation and promotion has been studied for some years [31-33]. PG´s may be involved in practically all aspects of tumorigenesis, and not only the tumor itself may have the capacity to synthetise PG´s (in quality and quantity different from normal tissue), but the tumor may also invite certain cells of the host defense mechanism to synthetise PG´s.

Bennett and associates [33] could correlate mammary tumor malignancy to the ***in vitro*** PG-like synthesis capacity of tumor biopsy material. Tumor PG synthesis capacity was found to be higher in malignant tumor tissue than in normal- or benign tumor tissue. Furthermore, hemostatic changes, induced by tumor TXA_2 synthesis, were hold responsible for changes in the ability of certain tumors to metastatise [34].

The second hypothesis, a mechanism by which host PG synthesis capacity is altered by tumor progression, has also been given

more attention recently [35]. Host macrophages or other monocytes, attracted by the tumor are known to be able to produce large quantities of PG's, predominantly PGE_2 [36]. High levels of PGE_2 may suppress NK cell activity and as such suppress one of the tumor host defense mechanisms [36].

In the present study, certainly the most important finding was that tumor growth in the rats receiving the diets containing 2 or 7 en% ω-3 fatty acids (EPA and DHA) was reduced, in comparison with those rats receiving diets containing 3 or 10 en% of omega-6 fatty acids, which is identical to the range of dietary polyunsaturated fatty acids in most Western diets. As diets C and D contained also 3 en% of linoleic acid, like diet A, the possibility of a protective effect by a relative essential fat deficiency was excluded. Since there was no difference between diets either containing 7% or 2% of omega-3 fatty acids, the quantity of EPA and DHA in these diets seems to be less important. The favorable effects of the diets containing EPA and DHA on tumor growth correlated with low plasma PGE_2 and TXB_2 values, and it seems therefore logical to suppose that the inhibited synthesis of these two PG's was in some way responsible for the favorable effect on tumor growth.

Despite the significant reduction in growth rate of the primary tumor, no favorable effect was seen on the number of tumor metastases developing. According to our protocol, tumors were removed, when a size of 20 mm was reached. The time span between inoculation and termination was therefore 5-6 days longer in rats on diets containing Menhaden oil (group C and D) than in rats without fish oil in their diet (groups A and B). When there is a positive correlation between the number of days after inoculation and the number of metastasis, this might as well have contributed to a higher number of metastases in groups C and D. However, we presume that the protocol as been used in this study has some benefits to a protocol using "time after inoculation" as a breaking point. It seems more reasonable to expect that the number of metastases is more strongly correlated with the tumor volume than with the number of days after inoculation.

In the present study, in tumor bearing animals, an effect on plasma $PGF_{1\alpha}$ and TXB_2 was observed. $PGF_{1\alpha}$ values were decreased whereas TXB_2 values were increased. However, these effects, although slightly less markedly, were seen in plasma of rats receiving γ-irradiated tumor tissue as well. This could indicate that not the tumor itself, but the tumor inoculation procedure might have been responsible for this. I.e. the effect of anesthesia, wound healing, and the host immunological reaction, etc. This was in accordance with the results of one of our

former studies using a malignant fibrosarcoma [37]. In that study also very markedly elevated plasma PG values were found in rats which bore γ-irradiated tumor tissue. However, γ-irradiated syngeneic spleen cells did nor show such an effect. Our conclusion was that a type of host immune response against tumor tissue antigens must have been the reason for the elevated plasma PG values, more than the tumor synthesis capacity.

It was striking that after primary tumor removal plasma PG values returned to pre-tumor inoculation values.

In the present study, no differences in cellular immune capacity could be established, therefore excluding the possibility that host immune capacity is altered at the time of initiation. Yet, later in the study, when tumor growth was more pronounced, differences in immune response may have occurred, but these will be hard to distinguish from differences in tumor size, metastases, etc. This was the reason for us not to determine cellular immune response later in the experiment.

Like others [38], we did not find any inhibitory effect in the ***in vitro*** platelet aggregation assay, of platelets from rats fed a EPA-rich diet or diets rich in omega-6 type of unsaturated fatty acids. However, in the earlier referred study [37], using a fibrosarcoma, changes were observed in maximum ADP-induced platelet aggregation, but these did occur rather "late" in the experiment. At that time tumors were large (>30 mm) and correlated with highly elevated plasma PGE_2 and TXB_2 levels, with values reaching 500 pg/ml and 1500 pg/ml, respectively. In the present study, the used method of ***in vitro*** platelet aggregation might not have been sensitive enough to detect the (certainly not more than very small) changes in platelet function induced by some of the experimental diets. However, ***in vivo*** measurements of bleeding time in rats, given a diet containing cod liver oil (rich in EPA), showed significantly prolonged bleeding times, when compared with rats on a diet containing sunflower seed oil (rich in linoleic acid) [16].

To conclude on the basis of this study that diets containing Menhaden oil might be beneficial to treat mammary tumor patients seems to be farfetched, but it appears that prolonged nutrition with diets containing EPA and DHA may have a favorable effect on this type of cancer incidence, such as was suggested by epidemiological studies in Greenland Eskimos and Japanese [9,11,25]. The mechanism for this favorable effect needs much more study, still one may conclude that host PG synthesis capacity -most important probably, the PGE_2 synthesis capacity- is the most valuable connective link between EPA and DHA rich diets on tumor reduction, although other mechanisms based on the

significantly altered physicochemical properties of cell membranes cannot be excluded sofar.

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Publication VIII

DIETS RICH IN FISH OIL CANNOT CONTROL TUMOR CELL METASTASIS

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DIETS RICH IN FISH OIL CAN NOT CONTROL TUMOR CELL METASTASIS

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Abstract

Rats fed diets containing different amounts of polyunsaturated fatty acids either of the omega-3 or omega-6 type, received cultured, syngeneic mammary tumor (BN472) cells i.v. Animals were sacrificed 2 weeks after tumor inoculation, and in the lungs the number of tumor foci were counted. No significant differences in the number of metastatic foci were observed in either one of the dietary groups.
Prostaglandin measurements in the supernatant of *in vitro* cultured tumor cells showed that the tumor cells could produce Thromboxane-A_2. By many investigators this tumor synthesis capacity was connected with tumor metastasis activity. Yet in our study, diets rich in Menhaden oil, with the known capacity to inhibit thromboxane synthesis, could not control tumor metastasis in this particular tumor model.

Introduction

The observation that diets rich in fish oil acted favorably on coronary heart disease of Greenland Eskimos, initiated an avalanche of publications on this subject [1-4]. This beneficial effect on cardiac heart disease is attributed to eicosapentaenoic acid and docosahexaenoic acid, which are present in concentrations up to 20% the fish oil types consumed by Eskimos [5]. EPA and DHA exert anti aggregatory actions, most probably by inhibiting the synthesis of the PG metabolic product TXA_2 [6,7].

As PG synthesis also seems to play an important role in tumor growth [8,9], it is logical that the role of fish oil-rich diets was investigated on tumorigenesis as well [10-12]. In these recent studies, favorable effects on tumor growth in animals, fed fish oil rich diets, were observed [10-12]. Apart from tumor growth, the metastatic spread of the tumor may also be influenced by, partially or selectively, blocking PG synthesis [13]. Therefore this particular aspect of carcinogenesis needs close attention as well.

In the present study tumor cells were inoculated intravenously in rats, which were on diets rich or poor in omega-3 and omega-6 (predominantly linoleic acid) unsaturated fatty acids. Two weeks after tumor inoculation the animals were sacrificed to determine the number of tumor foci in the lungs.

Materials and methods

Experimental design.: 40 BN/Bi inbred female rats (Zentral Institut für Versuchstierzucht, Hannover, Germany) entered the study at the age of 3 weeks (immediately after weaning). At arrival the animals were randomly selected into either one out of 4 diet groups, and immediately received the experimental foods. Every week the animals were weighed, and twice a week food consumption was established. Mammary adenocarcinoma was inoculated when the animals were 12 weeks of age, i.e. 9 weeks on diet. The experiment was terminated exactly 2 weeks after intravenous inoculation of tumor cells to establish the number of tumor foci in the lungs.

The rats were housed in Makrolon cages (5 rats/cage) on wood chips bedding, receiving food and water ad libitum. The animal

room was air-conditioned and had a controlled night-dark rhythm.
Tumor.: The tumor used in this study, originally classified as a malignant mammary adenocarcinoma (BN472), is a transplantable tumor routinely used in our studies on tumor growth and metastases [14]. When implanted subcutaneously, the tumor metastatises spontaneously from the primary site to regional lymph nodes and lungs. BN472 was grown as a suspension culture in 75 ml culture flasks containing 30 ml of RPMI 1640 (Biochrom K.G., Berlin, Germany) supplemented with 2% of foetal calf serum, at 37°C in a humidified atmosphere with 5% CO_2. The doubling time of the tumor cells was 10 hours.

Supernatant of tissue medium was harvested for PG determinations. PG values represent tumor cell synthesis of arachidonic acid metabolites per culture flask containing a mean (±SEM) of 3.0 (±0.3) $x10^5$ cells/ml over a period of 3 days. An other tumor, BN175, a malignant fibrosarcoma, routinely used in our laboratory as well [15], was studied as well. This tumor also has metastatic properties. A normal cell line (skin fibroblasts) was taken as an other reference. Culturing conditions of BN175 and fibroblasts were identical as was for BN472 cells, however the culture flasks with BN175 cells contained a mean (±SEM) of 5.3 (±0.4) $x10^5$ cells/ml, whereas fibroblast culture flasks contained 10 ml medium per flask and had a cell concentration (mean±SEM) of 3.6 (±0.2) $x10^5$ cells/ml (Table IV). Fibroblasts do not show any sign of malignancy, after intravenous injection of these cells, the animals live their normal lifespan.

When the animals were 12 weeks at age, $2.5x10^5$ vitale tumor cells were injected via the lateral tail vein, under light ether anesthesia. Two weeks after inoculation the animals were sacrificed and autopsied. Lungs were fixated in Bouin's fixative for microscopical examination of metastasis, which was performed by counting the metastases in a 3μm section of the left lung (longitudinal section). Tumor foci in the lung were scored semiquantitatively by classifying the microscopical count into the score: 0=0; 1-5=1; 6-10=2; 11-20=3; 21-30=4; >30=5.
Diets: Four experimental diets were studied, which all contained 30 en% of fat. The composition, with regard to their fatty acid content differed, by mixing various quantities of sunflower oil, cacaobutter, olive oil, linseed oil and/or Menhaden oil (Table I). The diets with 3 or 10 en% linoleic acid were named diet A and B respectively, and the diets with 7 or 2 en% of EPA + DHA were named diets C and D, respectively; they were prepared at Unilever Research Laboratories, Vlaardingen, The Netherlands. The diets were freshly prepared weekly in three portions. To

prevent peroxide formation, the diets were stored in sealed plastic bags, ***in vacuo***. The animals received the diets every Monday, Wednesday and Friday; at the same time the uneaten food was discarded.

Table I

CHARACTERISTICS OF THE STUDY DIETS

Composition of the experimental diets (g/1,000 Kcal)

Diet Compound	diet groups A-D
Casein	62.000
Minerals (Cas. '85)[a]	8.458
Vitamins (Vit. '70)[b]	1.000
Cellulose	15.000
Starch	134.286
Fatty acids	32.3

Sources of fatty acids (g/1,000 Kcal)

	diet group			
	A	**B**	**C**	**D**
cacao butter	24.946	13.656	-	7.204
sunflower oil	2.581	14.194	3.441	1.613
olive oil	4.839	4.409	0.108	13.118
linseed oil	-	-	3.226	-
Menhaden oil	-	-	25.376	10.323
total	32.366	32.259	32.151	32.258

Composition of the fatty acids (en %)[c]

	diet group			
	A	**B**	**C**	**D**
saturated fatty acids	15	10	10	10
monoenoic fatty acids	12	10	10	15
linoleic acid	3	10	3	3
EPA and DHA	0	0	7	2
total	30	30	30	30
P/S ratio[d]	0.2	1.0	1.0	0.5

a) In mg/1,000 Kcal: potassium chloride, 350; secondary magnesium phosphate, 956; primary potassium phosphate, 475; potassium bicarbonate, 719; calcium carbonate, 2014; trisodium citrate $2H_2O$, 711; manganese sulphate, 51.4; ferric citrate, 43.9; copper citrate, 4.7; zinc citrate, 12.5; potassium iodate, 0.07; tri-sodium dicitrate, 1630; magnesium acetate, 1490.

b) In mg/1,000 Kcal: choline (50%), 500; vitamin E (500 IU/g), 40; calcium silicate, 50; myo-inositol, 25; vitamin B_{12} (1,000 mg/kg), 5; vitamin A (325 IU/mg), 7.7; nicotinic acid, 5; (calcium)-panthothenic acid, 5; riboflavin, 1.5; thiamine mononitrate, 1.5; vitamin D (80 IU/mg); 3.125; vitamin K_3, (22.7%), 1; pyridoxine, 0.5; folic acid, 0.25; biotin, 0.05; sucrose, 354.375.

c) Established by gas chromatography.

d) P/S ratio = ratio between polyunsaturated fatty acids and saturated fatty acids.

Measurement of food consumption.: Twice a week, the quantity of fresh food given to the animals, was measured. Two days later food remnants were weighed again and the consumption per cage was calculated. By dividing this number by 10 (number of days x number of rats/cage) the average food consumption per rat per day was obtained.

Determination of prostaglandins: 6-keto-$PGF_{1\alpha}$, TXB_2 and PGE_2 were determined in culture supernatants, by means of a RIA, with the use of rabbit antisera (6-keto-$PGF_{1\alpha}$, Seragen, Boston, USA; TXB_2 and PGE_2, Institut Pasteur, Paris, France). After 3 days of culturing, the total content of a culture flask was centrifuged, after which samples of 1 ml of the supernatant were frozen at $-70^{\circ}C$ until they could be assayed.

Statistical evaluation.: A one way variance analysis was used to test the differences in the number of tumor foci in the lungs, body weight and food consumption. In case of significant differences (i.e., an F value indicating a probablity $<.05$), the test of Welch [16], was used to find which pair(s) of means were statistically significant different. A Yates analysis [17] was used to test the differences in the score of lung tumor foci.

Results

The animals accepted the experimental diets well; no significant differences in food consumption (Table II) were measured. Animals in group B (10 en% linoleic acid) had a significantly different growth rate ($p<.05$), however when a "percentile body weight increase" was calculated according to the formula

$$\frac{\text{body weight week (n) - body weight week (n-1)}}{\text{body weight week (n-1)}} \times 100,$$

no statistical significant differences were observed. This implies that the differences in body weight of the animals in group B were due to their initial differences (Table III).

Peroxidation was measured in food samples, which were left at room temperature for 3 days (the maximum of exposure to air and room temperature). Values were 4.2, 12.1, 32.2, 15.2 milli equivalent O_2/kg fat, for diet groups A, B, C, and D, respectively. The low values indicate that the degree of peroxydation was only minimal.

Table II

Food comsumption/rat/day (grams)[ab]

wks of age	diet group A	B	C	D
4	5.6 (.16)	5.7 (.17)	5.2 (.15)	5.4 (.16)
5	6.0 (.18)	7.1 (.21)	6.2 (.18)	6.5 (.19)
6	6.3 (.18)	6.5 (.19)	6.2 (.18)	6.3 (.18)
7	6.3 (.18)	6.8 (.20)	6.3 (.18)	6.4 (.19)
8	6.5 (.19)	6.6 (.19)	6.7 (.19)	6.5 (.19)
9	7.3 (.21)	7.2 (.21)	6.9 (.20)	7.1 (.20)
10	7.4 (.22)	7.4 (.22)	6.5 (.19)	6.5 (.19)
11	7.7 (.22)	7.5 (.22)	7.1 (.20)	7.0 (.20)
12	7.3 (.21)	7.0 (.20)	6.7 (.19)	6.8 (.20)
13	7.3 (.21)	7.3 (.21)	6.9 (.20)	6.7 (.19)

a) See for composition experimental diets, Table I. Values given in parenthesis, SEM.
b) No statistical significant differences in food consumption in any of the dietary groups.

Table III

Body weight of experimental animals (grams)[ab]

wks of age	diet group A	B	C	D
3	46 (2.0)	48 (0.7)	46 (2.0)	45 (1.7)
4	65 (2.1)	69 (0.9)	64 (2.2)	65 (1.7)
5	78 (2.8)	83 (1.5)	78 (2.4)	81 (2.0)
6	88 (3.3)	95 (2.5)	90 (2.5)	92 (2.0)
7	100 (3.4)	110 (3.5)	101 (3.3)	104 (2.6)
8	108 (4.5)	118 (3.7)	113 (3.1)	114 (3.0)
9	118 (4.4)	127 (4.1)	120 (3.4)	122 (3.0)
10	126 (4.2)	137 (4.1)	127 (3.2)	130 (3.1)
11	136 (3.9)	146 (4.2)	134 (3.4)	137 (3.1)
12	141 (3.9)	150 (4.1)	139 (3.4)	142 (3.2)
13	146 (4.1)	156 (3.6)	146 (3.8)	147 (3.2)

a) See for composition experimental diets, Table I. Values given in parenthesis, SEM.
b) Body weight of the animals in diet group B is significantly increased ($p < .01$) in comparison with diet group A, C and D.

The results of the PG measurements in the supernatant of cell cultures (Table IV) showed that both tumor BN472 and BN175 had no detectable PGE_2 and prostacyclin synthesis, however, synthesis of TXA_2 could be demonstrated. PG synthesis of the fibroblasts in tissue culture was significantly different from that of the mentioned two tumor cells lines. Not only was synthesis of PGI_2 and PGE_2 clearly present, but also TXA_2 values were significantly higher.

The scores of the tumor foci in the lungs are given in Table V. Differences were minimal (diet D has a relative "favorable" score) and were not statistically significant.

Table IV

PG values in supernatant of tissue culture (pg/10^6 tumor cells)[a]

	PGE_2	6-keto-$PGF_{1\alpha}$	TXB_2
tumor BN472[b]	<10	<10	641 (104)
tumor BN175[b]	<10	<10	999 (103)
fibroblasts	1170 (89)	3965 (236)	1908 (255)

a) Number of measurements = 6; values given in parenthesis, SEM.
b) See for tumor characteristics, Materials and Methods.

Table V

Number of animals having a certain score of tumor foci in the left lung[ab]

tumor score	diet group A	B	C	D
0	0	0	0	0
1-5	3	4	3	7
6-10	3	1	4	2
11-20	3	4	3	0
21-30	0	1	0	0
<30	1	0	0	1

a) See for composition experimental diets, Table I.
b) No statistical significant differences in the number of metastasis.

Discussion

EPA- and DHA-rich diets have important inhibitory effects on hemostasis, possibly by changing PG synthesis [4,18]. The possible significance of this with regard to tumor initiation and promotion has not been evaluated sufficiently, but some studies on this subject have been completed recently [10,19]. So far most of these studies were concerned with tumor growth itself, and it was surprising that the process of metastasis received only little attention. This is surprising as many investigators link the ability of a malignant tumor to metastatise to its capacity to synthetise "procoagulant factors", most probably TXA_2 [13]. By this mechanism, indomethacin or other non steroid anti inflammatory agents (NSAIA) could reduce tumor growth as well as the number of metastasis [20-22]. Because EPA and DHA rich diets act more or less similar on hemostasis as NSAIA's do, the study of the effect of fish oil rich diets on this aspect of the carcinogenesis was certainly justified.

The results of the present study showed that the expected favorable effect on the "take" of tumor cells was not present. In an earlier study by us, using the same tumor and dietary regimen, in a model in which the tumor was implanted subcutaneously, no differences in spontaneous dissemination of the tumor was could be found as well, although differences in tumor growth were observed [Kort, W.J., ***et al.***:to be published].

In this study and in those previous carried out by us and by others [7], the effect of fish oil rich diets on PG synthesis and hemostasis could be shown. Plasma PGE_2 and TXA_2 values were significantly decreased in those rats fed the diets rich in Menhaden oil [Kort, W.J., ***et al.***:to be published]. In experiments of Hornstra et al, the in vivo bleeding time was significantly prolonged [7]. This implies that if the theory that hemostatic changes play a role in tumor metastasis, as for instance was postulated by Honn et al [23], is correct, the animals receiving the diets rich in Menhaden oil, should have a reduction of the number of metastatic foci in the lungs. However in the present study we could not support this hypothesis.

A necessary condition for a possible favorable effect of diets or drugs with TXA_2-blocking properties will depend of course on the capacity of the tumor to produce TXA_2; this condition was fulfilled in this study. In the supernatant of the cell cultures of tumors with metastatising capacities, TXB_2, the stable degradation product of TXA_2, was present. An other interesting finding was that a non malignant fibroblast cell line was not very much different from our two tumor cell lines with regard to their capacity to synthetise TXA_2 (except for quantitative differences), but was for its capacity to poduce PGI_2 and PGE_2. Therefore the metastatic ability of a certain tumor type does not seem to be exlusively dependent of the capacity of that cell to produce TXA_2, but may be more related to the ratio of all PG's synthetised. Which latter implies that blocking the TXA2 synthesis alone, would not do much good with regard to prevention of tumor metastasis, although the first results with TXA_2 synthetase blockers and TXA_2 receptor blockers in some tumor metastasis models showed a significant regression of the tumor spread [24-26].

The importance of the observed quantitative differences in tumor TXA_2 synthesis is difficult to assess. Although there are differences in tumor malignancy between tumor BN472 and BN175 (BN175 is the more agressive one; tumor growth rate is higher [14]), one may conclude that there is a correlation between tumor malignancy and tumor TXA_2 synthesis, however the fact that also the fibroblast cell line could produce considerable amounts of

TXA_2 militates against such a connection.

The model of intravenous tumor inoculation is a model in which the process of tumor cell "take" can be studied. Certainly for processes involving changes in hemostasis, such as been studied here, the model of intravenous tumor inoculation seems to be the model of choice. However, we have to keep in mind that in this model only one aspect of the multistaged process of tumor metastasis is involved and that to study the other steps of a metastatising tumor cell a spontaneous metastatising tumor is necessary.

So far we may conclude that despite the fact that, with fish oil-rich diets, conditions can be achieved which alter hemostasis, no inhibitory effect was seen on the number of tumor foci in the lungs, after intravenous tumor inoculation.

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Publication IX

CONVERSION OF DIETS AT TUMOR INDUCTION SHOWS THE PATTERN OF TUMOR GROWTH AND METASTASIS OF THE FIRST GIVEN DIET

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CONVERSION OF DIETS AT TUMOR INDUCTION SHOWS THE PATTERN OF TUMOR GROWTH AND METASTASIS OF THE FIRST GIVEN DIET

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Abstract

In a previous study, significant differences in the growth rate of a transplantable mammary adenocarcinoma were observed between rats, receiving a diet rich in saturated fatty acids (lard, diet A), and rats on a diet, in which for partly the saturated fat was replaced by polyunsaturated fatty acid of the ω-3 type (Menhaden oil, diet B).

In the present investigation, it was our aim to study tumor growth in rats on diet A and B, as well in rats, that had, at tumor inoculation, a change of diets, from diet A to diet B.

Tumor growth of rats receiving diet A throughout the whole experiment was the same as in those rats that had a conversion of diets. The data shows that the observed inhibiting effect of diet B on tumor growth could not be obtained when this diet was given exclusively after tumor inoculation.

Introduction

Marked differences in incidence of colon and mammary tumors are seen between Western countries and Asian or African countries [1-3]. For these differences environmental conditions are attributed. Life style studies amoung the main ethnic groups in Hawaii showed a wide range in cancer incidence rates [4]. Other important findings in these studies were the positive correlation between the incidence of certain tumor types (e.g. breast cancer) and saturated fat consumption. Moreover, tumor incidence rates of second generation Hawaii inhabitants, with adopting a more Westernized food consumption pattern, tended to change in the direction of those of Hawaii born Caucasians [4].

Many constituents of the diet were mentioned that could either promote or inhibit carcinogenesis [1,5,6]. On the basis of these data, or from anecdotal sources, several types of "health food" were marketed [7-10]. The effects of these diets on tumor prevention is hard to assess and remains uncertain, although some investigators claim to have found good correlations for, e.g. high vitamin C, high fiber, or low fat with a decreased tumor incidence [9,10].

A "therapeutic" effect of certain diets on cancer has been proposed also [11,12]. Food constituents such as vitamin C and fiber were given in excess to cancer patients, not in order to counteract nutritional inadequacies as a result of cancer cachexia, but in order to cure the cancer [12]. Also "macrobiotic" diets have been advocated [11]. These ideas have hardly any scientific foundation and were subject to much criticism [13].

Conclusive evidence from epidemiological studies in man should be obtained from studies in experimental animals. Many such studies were carried out in rats, for instance confirming the epidemiologically established correlation between high fat intake and increased mammary tumor incidence [14-17]. Although many dietary constituents have been studied, the quantity and quality of dietary fat, received most attention. In one of such studies by us, we could show that animals receiving diets, enriched in Menhaden oil, had an inhibited tumor growth when compared with rats receiving diets, rich in saturated fat or enriched in linoleic acid [Kort, ***et al***, in preparation].

This finding was taken as a starting point for the present study. In this study we were especially interested in the ability of the diet rich in Menhaden oil to inhibit tumor growth or tumor metastasis when the diet was given exclusively after

tumor inoculation, that is to say as a therapy to control tumor progression.

Materials and methods

Experimental design.: 75 BN/Bi inbred female rats (Zentral Institut für Versuchstierzucht, Hannover, Germany) entered the study immediately after weaning (3 weeks of age). At arrival the animals were randomly divided into 3 groups, 2 of which first received diet A (Group A-A; Group A-B) while one group received diet B continuously (Group B-B). Every week the animals were weighed while at the same time food consumption was established. Tumor inoculation with BN472 (a transplantable mammary adenocarcinoma) was performed when the animals were 12 weeks of age, i.e. 9 weeks on experimental diet. On the day of tumor inoculation, the diet of the animals in Group A-B was converted from diet A to diet B. 18 days after tumor inoculation primary tumors were removed, and 13 days therafter the experiment was terminated by sacrificing the animals. Housing conditions were controlled and identical in all groups.

Tumor inoculation.: BN472, a transplantable mammary adenocarcinoma, was implanted subcutaneously in the right flank of the rat under light ether anesthesia. 18 days therafter, the primary tumors were surgically removed, again under ether anesthesia. 13 days after primary tumor removal the animals were sacrificed and underwent autopsies. Inguinal, axial and paraaortal lymph nodes were examined macroscopically for tumor metastasis, and the lungs were fixated in Bouin´s fixative for microscopical examination of metastasis. This was performed by counting the metastases in one 3μm section of the left lung (longitudinally). Lung metastases were scored semiquantitatively by classifying the microscopical count into the score: 0=0; 1-5=1; 6-10=2; 11-20=3; 21-30=4; >30=5.

Measurement of food consumption.: Once a week the quantity of fresh food given to the animals was weighed. Two days later the food remnants were weighed again and the consumption per cage, in which 5 animals were housed, was calculated. By dividing this number by 10 (number of days x number of rats/cage) the mean food consumption per rat per day could be calculated.

Diets: The experimental diets, prepared at Unilever Research Laboratories, Vlaardingen, The Netherlands, contained 30 en% of fat but were differently composed with regard to their fatty acid

content (Table I). The diets were freshly prepared weekly in three portions: one for 3 days, the other two for 2 days. To prevent peroxyde formation, the diets were stored in sealed plastic bags, ***in vacuo***. The animals received the diets every Monday, Wednesday and Friday; at the same time discarding the uneaten food.

Table I

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sunflower oil	2.581	3.441
olive oil	4.839	0.108
linseed oil	-	3.226
fish oil	-	25.376
total	32.366	32.151

Composition of fatty acids (en %)[c]

	diet group A	diet group B
saturated fatty acids	15	10
monoenoic fatty acids	12	10
linoleic acid	3	3
EPA and DHA	0	7
total	30	30
P/S ratio[d]	0.2	1.0

a) In mg/1,000 Kcal: potassium chloride, 350; secondary magnesium phosphate, 956; primary potassium phosphate, 475; potassium bicarbonate, 719; calcium carbonate, 2014; trisodium citrate $2H_2O$, 711; manganese sulphate, 51.4; ferric citrate, 43,9; copper citrate, 4.7; zinc citrate, 12.5; potassium iodate, 0.07; tri-sodium dicitrate, 1630; magnesium acetate, 1490.

b) In mg/1,000 Kcal: choline (50%), 500; vitamin E (500 IU/g), 40; calcium silicate, 50; myo-inositol, 25; vitamin B_{12} (1,000 mg/kg), 5; vitamin A (325IU/mg), 7.7; nicotinic acid, 5; (calcium)-panthothenic acid, 5; riboflavin, 1.5; thiamine mononitrate, 1.5; vitamin D (80 IU/mg); 3.125; vitamin K_3, (22.7%), 1; pyridoxine, 0.5; folic acid, 0.25; biotin, 0.05; sucrose, 354.375.

c) Established by gas chromatography.

d) P/S ratio = ratio between polyunsaturated fatty acids and saturated fatty acids.

Statistical evaluation.: A Student t-test was used to test the differences in body weight, food consumption, and tumor growth rate. A Yates analysis [18] was used to test the differences in the score of metastasis. Differences were considered statistically significantly different, when $p \leq .05$.

Results

The animals accepted the experimental diets well; no significant differences in food consumption were observed (Figure 1). Also differences in body weight were not statistically significant (Figure 2).

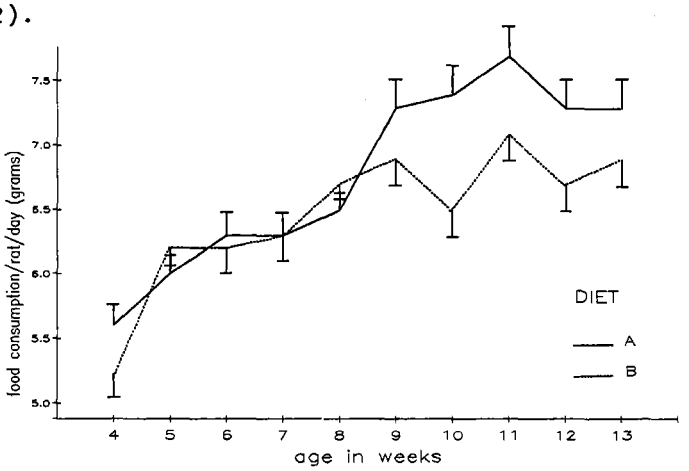

Fig. 1. – Determination of food consumption, no statistically significant differences between animals fed diet A or diet B.
See for composition of the experimental diets, Table I.

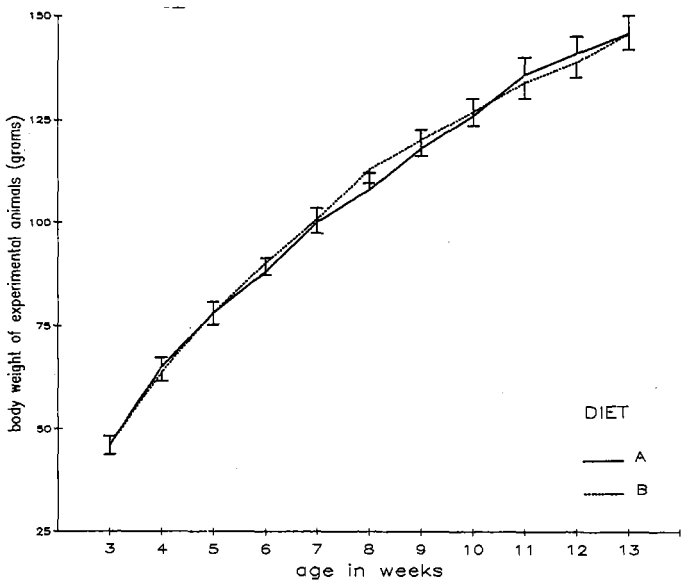

Fig. 2. – Determination of body weight, no statistically significant differences between animals fed diet A or diet B.
See for composition of the experimental diets, Table I.

Peroxidation was measured in food samples, which were left at room temperature for 3 days (the maximum of exposure to air and room temperature). Values were 4.2 and 32.2 milli equivalent O_2/kg fat, for diet groups A and B, respectively. The low values indicate that peroxydation was only minimal, which is agreement with the observed similar growth rates of the two dietary groups.

A number of animals had to be excluded from further evaluation because the tumor did not show any growth at day 18: 5, 3 and 7 rats in Group A-A, Group B-B, and Group A-B, respectively.

The differences in tumor growth rate and the scores of the metastatic foci in the lungs can be seen in Table II. Differences were only observed for tumor growth between groups A-A and A-B on the one hand, and Group B-B, on the other (Group B-B vs Group A-A and Group A-B, statistically significant, $p<.05$). In the score of tumor foci in the lungs, differences were minimal and were not statistically significant.

Table II

Mean tumor growth rate and mean score of metastasis[a]

	dietary group		
	A-A	**B-B**	**A-B**
mean tumor growth rate[c]	15.4 (0.6)	21.5[b] (2.3)	15.5 (0.8)
tumor metastasis score[d]			
0	5	4	4
1-5	5	6	5
6-10	2	2	2
11-20	3	4	4
21-30	-	2	-
>30	4	4	3
No. of rats with pos. ly. nodes/ total No. of rats	4/20	2/22	5/18

a) See for composition of experimental diets, Table I. Values given in parenthesis, SEM.
b) Difference statistically significant vs diet group A-A and A-B ($p < .05$).
c) Mean tumor growth rate = Mean number of days before the tumor reached a diameter of 20 mm.
d) Differences in tumor spread to the lungs were not statistically significant.

Discussion

The importance of a therapeutic use of nutritional factors to cope with dietary inadequacies as a result cancer cachexia has been fully recognized [19,20]. The patient's condition might improve, and a better response to antineoplastic drugs or an improved tolerance to their side effects has been observed [21,22].

On the other hand, certain dietary regimens have been also suggested as alternative treatment modalities to the orthodox therapeutic approach to cancer [11,13]. This means that such diets are advocated as a sole therapy for cancer. Many of such unorthodox therapies exist, and sometimes receive much attention in the popular press [23-25]. Generally, the composition of these diets lack any scientific basis: controlled trials have not been performed and ***in vitro*** or ***in vivo*** research in experimental animals is lacking.

One of the components also receiving attention in circles of the practitioners of alternative cancer management, as a dietary component with cancer promoting properties, was animal fat [24-25]. Siguel [24] gives a theoretical explanation for the use of vegetarian diets for its "cancerostatic" effect. Shortly, the vegetarian diet would deprive neoplastic cells of higher chain fatty acids and of the enzym 6-desaturase. Such a depletion would result in altered membrane fluidity, altered membrane transport properties, the ability to reproduce, etc., and by these means decrease neoplastic cell survival.

Fish oil enriched diets, as were used in the present study were given as a tumor controlling diet, with more or less the same theoretical background in mind. Also polyunsaturated fatty acids present in fish oil (eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) alter membrane fluidity, arachidonic acid metabolims, etc. [26]. Its potential use as a cancerostatic component in nutrition was first suggested from epidemiological studies in man [27] and later confirmed in experimental animals by Karmali ***et al*** [28], and by us [Kort, ***et al***, in preparation]. The conclusion from these studies was that the diets rich in fish oil, when given before and after tumor inoculation of a transplantable mammary adenocarcinoma, could reduce the tumor growth. However, due to the dietary protocol used in these studies it was not clear whether the diets exhibited their beneficial effect before or after tumor inoculation.

The results of the present study show that a favorable effect

can be obtained as a result of the dietary status of the animal before tumor inoculation, and that there is no "therapeutic" effect of the diet. Since the mean period to achieve a tumor size of 20 mm diameter was similar in diet groups A-A and A-B and different from B-B.

Apart from its inhibitory effect on mammary tumor growth, diets enriched with fish oil may also be able to control tumor cell metastasis. The theoretical background is the ability that EPA and DHA alter platelet function by reducing Thromboxane-A_2 synthesis and by these means may interfer with tumor cell platelet interactions, a mechanism which is considered to be important for tumor cell metastasis [29-32].

However, in the present study, no statistically significant differences in the number of metastatic foci in the lungs were observed. Therefore we cannot support a possible therapeutic approach for reducing tumor metastasis via fish oil rich diets.

We therefore conclude that, on the basis of the presented data, no therapeutic effect can be expected of fish oil rich diets, neither on controlling tumor growth, nor in controlling tumor metastasis. A possible beneficial effect on tumor growth can be achieved only when the diet rich in fish oil is given ample time before inoculation of the tumor.

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Publication X

GROWTH OF AN IMPLANTED FIBROSARCOMA IN RATS IS ASSOCIATED WITH HIGH LEVELS OF PLASMA PROSTAGLANDIN-E_2 AND THROMBOXANE-B_2

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GROWTH OF AN IMPLANTED FIBROSARCOMA IN RATS IS ASSOCIATED WITH HIGH LEVELS OF PLASMA PROSTAGLANDIN-E_2 AND THROMBOXANE-B_2

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ABSTRACT

Growth of BN175, a malignant fibrosarcoma, was correlated with high plasma TXB_2 and PGE_2 levels. This statistically significant increase was first detected 17 days after inoculation of the tumor, at which time the tumors were 20 mms in diameter. A further increase in tumor size was associated with still higher PGE_2 and TXB_2 values. At the same time, progressive alterations in platelet function, as measured by ADP-induced platelet aggregation, were observed. 6-keto-$PGF_{1\alpha}$ levels remained normal throughout the whole experiment.

It was concluded that tumor growth was associated with changes in PG synthesis and platelet function, although it remains unclear whether these changes were caused by some host immunological response towards the tumor or were predominantly the result of tumor PG-synthesis.

INTRODUCTION

Like normal cells, cancer cells synthetize PG's[1]; however, quantitative and qualitative differences in PG-synthesis between malignant tumor cells, on the one hand, and benign tumor or normal cells, on the other, have been observed (1-3). For instance in several types of malignant tissue, both in humans and experimental animals, high synthesis of the PGE type of prostaglandins has been found (1-3). In other studies, PGI_2 and TXA_2 synthesis were also found to be higher in tumor tissue than in normal or hyperplastic tissue (3-5).

Some tumor associated disorders have been related to altered PG synthesis; e.g. hypercalcemia, often to be found in breast tumor patients, and diarrhoea in a patient with a medullary thyroid carcinoma, were

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[1] The following abbreviations were used: PG's = prostaglandins, TXB_2 = thromboxane B_2, TXA_2 = thromboxane A_2, PGE_2 = prostaglandin E_2, PGI_2 = prostaglandin I_2 = prostacyclin, 6-keto-$PGF_{1\alpha}$ = 6-keto prostaglandin $F_{1\alpha}$, ADP = adenosine diphosphate, RIA = radioimmuno assay.

attributed to enhanced PG synthesis ativity (6,7). Increased PG synthesis has also been connected with metastasis (8). In particular Honn and his group showed that alterations of the TXA_2/PGI_2 ratio in favor of TXA_2 correlated with an increase of the number of metastatic foci in the lungs of rats in which tumor cells were inoculated intravenously (9).

Because of its importance in certain tumor associated complications and possibly its influence on tumor metastatic pattern, PG-synthesis or synthesis capacity might be a useful tool to monitore tumor status in patients. Consequently, Bennett *et al.* (10) and also Karmali *et al.* (3), and later many others (2), measured PG synthesis in specimens of tumor tissue obtained at surgery and found evidence that, for instance, breast carcinomas produced more PG's than normal tissue and that the level of PG synthesis could be related to staging of the disease.

The present study was undertaken to elucidate further the relationship between tumor growth and PG synthesis. Most importantly, the study was initiated to determine whether plasma PGE_2, TXA_2 or PGI_2 values as measured by RIA assay could be used as a parameter to follow tumor progresssion, and if blood platelet aggregatory changes could be established in tumor bearing rats, which could be associated with either tumor progression or changes in plasma PG-values.

The tumor used in this study has been used in several other investigations; it metastases from site to regional lymph nodes and lungs and appears to be sensitive to aspirin therapy (11,12).

MATERIALS AND METHODS

Experimental design and animals - Inbred Brown Norway (BN) female rats weighing 125-175 were used. Tap water and rat chow (Hope Farms AMII, Woerden, The Netherlands) were given *ad lib.* For every determination of either PG's or platelet aggregation, 5 different rats were taken, as we considered frequent blood sampling in the same rat would have too much influence on consecutive measurements. When blood samples were taken from tumor bearing animals, at the same time, they were also taken from sham treated control rats. Control values for PG and platelet aggregation were measured in animals in which pieces of tumor tissue (that had been irradiated with 15000 Rads γ-irradiation) were inoculated. Moreover another group of animals, in which irradiated isogeneic spleen cells were inoculated, served as controls to establish the influence of the inoculation procedure itself on the parameters used. The environmental conditions of the rats under study were strictly controlled and for all animals, either experimental or sham, identical.

Tumor - The tumor used in this study was BN175, a malignant fibrosarcoma, classified as a non immunogenic undifferentiated mesenchymal tumor (12). In this study the tumor was inoculated in the flank, by putting a small piece of vital tumor tissue (about $5mm^3$) under the skin. Tumor size was measured twice weekly using calipers and determining the diameter by calculating the mean of length and width.

Tissue culture - Cells of tumor BN175 are kept in tissue culture in RPMI 1640 medium to which 5% foetal calf serum has been added. Twice a week the tumor, which grows very rapidly (doubling time 24 hrs), is further diluted into a next passage. At the time of the experiment the tumor was in its 20th passage. PG synthesis by BN175 in tissue culture was measured in the supernatant just before another passage.

In vitro platelet aggregation - Platelet aggregation was carried out according to the procedure as described earlier (12) using the Chronolog-Whole Blood Aggrometer (Chronolog Corp., England). Heparinized (10 U/ml) whole blood samples were diluted 1:1 with 0.9% saline, ADP (10 μM) was added to induce platelet aggregation. 10 minute recordings of the induced changes in electrical resistance (impedance, values given in 'Ω') were analysed by an Olivetti M24 PC, which calculated the maximum aggregation. Platelets were counted on a TOA Platelet Counter PL100.

Determination of PG's - PG's were determined in blood plasma by means of a RIA, with the use of rabbit antisera (6-keto-$PGF_{1\alpha}$, Seragen, Boston, USA; TxB_2 and PGE_2, Institut Pasteur, Paris, France; cross reactivity of these antisera with each other was <.1% at 50% B/Bo). RIA's were carried out in plasma from the same blood sample in which the platelet aggregations were performed. To a blood sample of 2.5 ml, 250 μl 2.8% of EDTA and 10 μl 0.3% of indomethacin was added immediately after bleeding the animals. Within half an hour thereafter, the samples were centrifuged and plasma was stored at -70°C until they were assayed, according our routine procedure which was described earlier (13).

Statistics - Statistical evaluation of the differences were tested by using a Student's t-test. A difference was considered statistically significant when the calculated P-values were <.05.

RESULTS

34 out 35 animals, in which tumor BN175 was inoculated showed tumor growth. The one animal (killed on day 9), in which tumor inoculation was not successful, was excluded from further evaluation. 2 animals died before the intended day of sacrifice (day 30), from lung metastases.

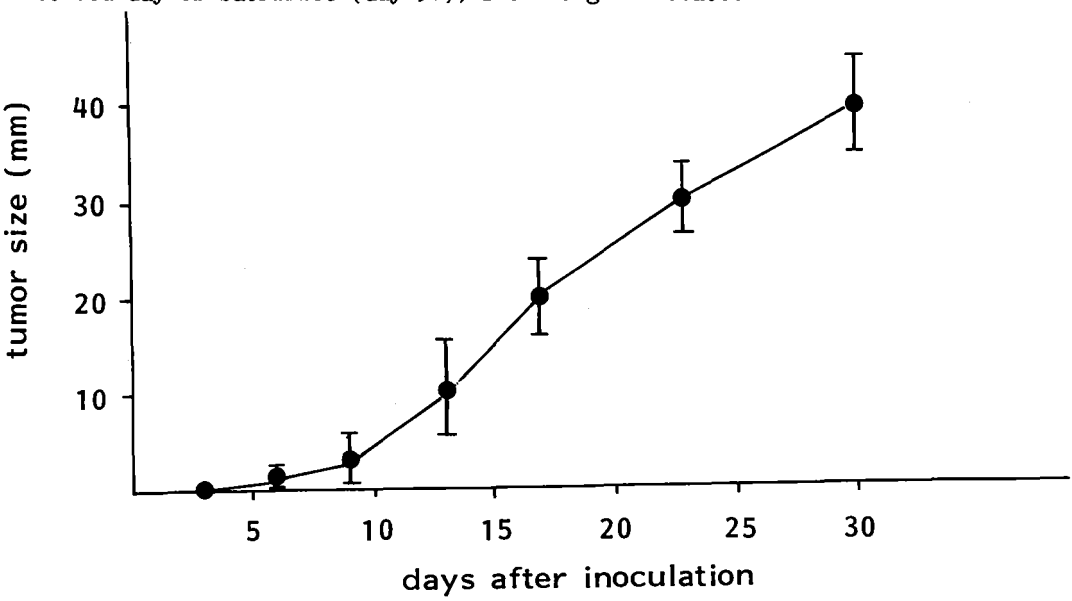

Fig. 1. Tumor growth after inoculation of BN175; n=5, vertical bars represent ±SEM.

Animals, in which γ-irradiated pieces of tumor or spleen tissue were inoculated, did not show any visible sign of tumor growth or inflammatory reaction.

Tumor growth pattern is shown in Fig. 1. First detectable tumor growth could be observed 6 days after inoculation, while at 30 days, the tumor reached a mean diameter of 40 mm. Tumors reaching 30 mm or more impeded the animal's condition markedly; such tumors often ulcerated, and were partly necrotic, the animals being weakened by blood loss and inflammation.

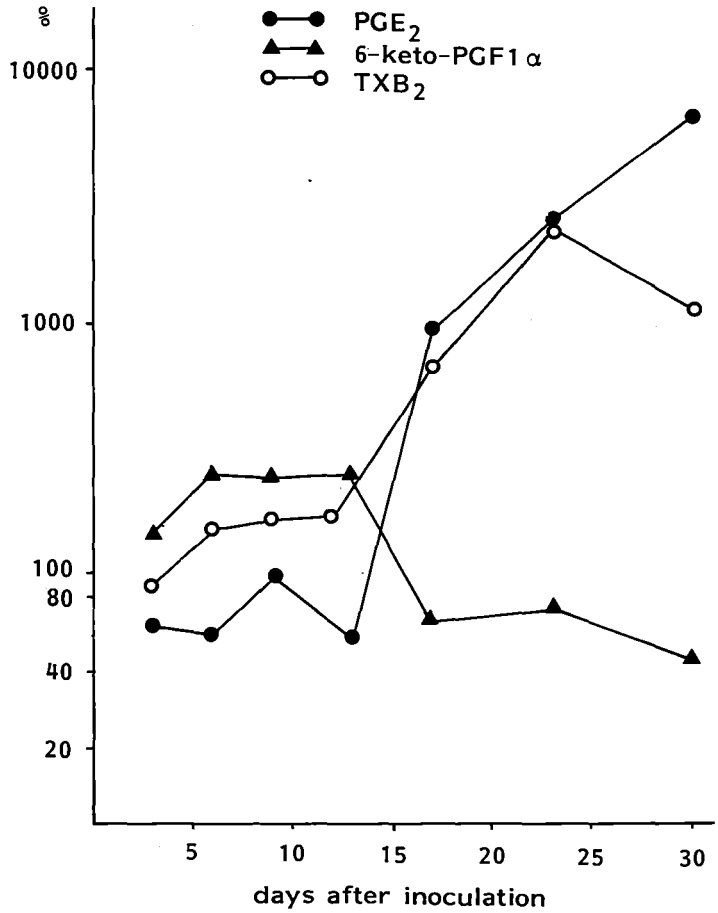

Fig. 2. Plasma PG values after inoculation of BN175; values are given as percentage of values of rats, in which γ-irradiated pieces of tumor were inoculated; see for reference values and for SEM, Table I.

Table I and Fig. 2 show the results of PG determinations. Because it is to be expected that operation trauma and immunological reactions may influence PG values, in Fig. 2, PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 are depicted as percentage of control (=γ-irradiated tumor) values. Until day 13, values

Table II. Plasma PG values (pg/ml) after inoculation of γ-irradiated spleen cells. Results show mean, in parenthesis: SEM.

day	PGE_2	6-keto-$PGF_{1\alpha}$	TXB_2	n
3	86.6 (21.5)	53.0 (7.9)	76.0 (15.3)	5
6	63.2 (7.0)	58.8 (20.4)	64.4 (6.3)	5
13	36.2 (8.0)	52.0 (9.0)	29.4 (4.8)	5
unoperated control values				
	27.1 (6.4)	71.1 (12.4)	34.4 (7.1)	12

At the same time that significant changes occurred in PG levels, ADP induced platelet aggregation altered as well (Table III, Fig. 3). The progressive decrease in platelet function (except for the non-significant rise at day 9), resulted in statistically significant ($p<.05$) inhibition of platelet aggregation from day 13 onwards.

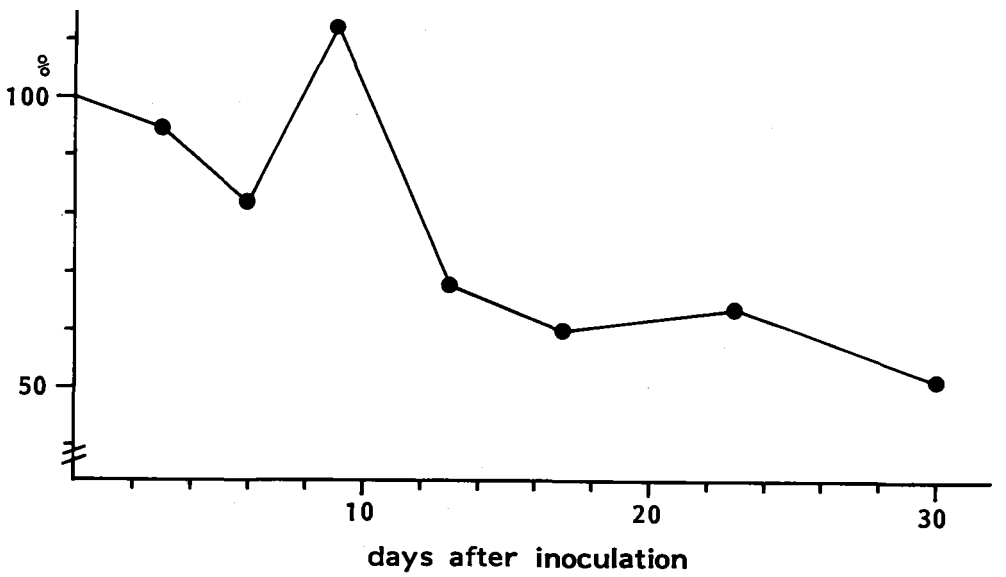

Fig. 3. Percentage of maximum aggregation achieved with 10 μmol ADP, after inoculation with tumor BN175; 100% = aggregation of blood taken from animals, in which γ-irradiated pieces of tumor were inoculated; see for reference values and SEM, Table III.

remain around the 100% level. Thereafter plasma PGE_2 and TXB_2 increased and reached values 10-50 times the values of sham operated animals. These differences are statistically significant ($p<.05$). 6-keto-$PGF_{1\alpha}$ values remain normal: from day 13 onwards, there was even a slight, but not statistically significant decrease in PGI_2 activity.

The tumor inoculation procedure itself causes changes in PG-levels as well (Table I). During the first 2 weeks after inoculation of a γ-irradiated piece of tumor tissue, values of PGE_2 and TXB_2 were increased, when compared with unoperated control values (Table II). The values reached a maximum 9 days after inoculation. To determine what part of the PG values in the group of rats with γ-irradiated tumors could be ascribed to operation trauma and what part to some type of host anti-tumor reaction, γ-irradiated spleen cells were inoculated as well. PG values determined in plasma from these rats showed shortly after inoculation a slight increase in PGE_2 and TXB_2 values: however, this increase disappeared shortly therafter and values were normal again 2 weeks after inoculation.

Table I. Plasma PG values (pg/ml) after tumor inoculation of tumor BN175 (TUM) and BN175 γ-irradiated with 15000 Rads (γTUM, ***values in Italics***). Results show mean, in parenthesis: SEM.

day	PGE_2		6-keto-$PGF_{1\alpha}$		TXB_2		n
	TUM	γTUM	TUM	γTUM	TUM	γTUM	
3	90.6 (40.5)	*147.0 (61.9)*	45.2 (6.9)	*32.2 (3.5)*	81.2 (20.9)	*94.9 (17.3)*	5/5
6	25.0 (12.2)	*45.0 (4.7)*	33.2 (4.4)	*13.4 (6.5)*	65.6 (7.5)	*44.0 (2.7)*	5/5
9	356.0 (205.1)	*374.2 (206.9)*	61.0 (17.4)	*25.3 (10.2)*	306.0 (163.3)	*185.0 (58.2)*	4/5
13	111.0 (16.6)	*204.0 (42.4)*	54.4 (6.3)	*21.8 (4.1)*	133.8 (49.8)	*79.6 (12.9)*	5/5
17	151.2 (62.6)	*16.0 (5.7)*	30.4 (7.6)	*48.2 (1.6)*	141.8 (38.3)	*20.9 (10.6)*	5/5
23	476.0 (117.3)	*38.0 (5.3)*	39.8 (13.2)	*56.6 (8.9)*	1222.6 (472.7)	*51.6 (3.7)*	5/5
30	521.0 (48.4)	*8.0 (4.1)*	21.3 (10.7)	*47.0 (8.4)*	1618.3 (228.7)	*13.6 (8.3)*	3/5

PG determinations in supernatants of cell cultures of tumor BN175, 3 days after seeding, did not show any detectable quantities of either PGE_2 or 6-keto-$PGF_{1\alpha}$, but demonstrable levels of TXB_2 could be established. Determinations in 6 different culture flask containing 1.4×10^7 cells in 30 ml of culture medium, showed a mean (±SEM) of 529.3 (±54.4) pg TXB_2 per 1 ml culture medium.

Values, calculated as a percentage of those values obtained from rats, in which γ-irradiated tumor cells were inoculated, finally leveled off at approximately 60%. Other characteristics of ADP aggregation, such as lag time, shape changes or reversibility of the aggregation, did not show a significant alteration in any of the experimental groups. The number of platelets was not affected by tumor growth and was as such not correlated with the established changes in the *in vitro* platelet aggregation.

Table III. Values of maximum aggregation (values in Ω) after inoculation of tumor BN175 (TUM) and BN175 γ-irradiated with 15000 Rads (γTUM), achieved with 10 μmol ADP, in parenthesis: SEM.

day	mean (SEM)	n	mean (SEM)	n
	TUM		γTUM	
3	17.2 (2.4)	5	18.2 (1.9)	5
6	16.8 (3.0)	5	20.4 (1.7)	5
9	18.1 (1.5)	5	16.0 (1.0)	5
13	17.0 (1.2)	5	25.1 (2.7)	5
17	10.7 (1.1)	5	17.9 (1.0)	5
23	11.5 (4.5)	5	18.1 (1.1)	5
30	9.1 (1.6)	5	17.3 (0.9)	5

DISCUSSION

While most previous studies, in both humans and experimental animals, reporting elevated PG synthesis, were based on measurements of PG values extracted from tumor biopsy material (2,14,15), this report demonstrated that PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 determined in rat plasma also showed considerable alterations in tumor bearing rats. Mean levels of TXB_2 and PGE_2 were markedly increased when compared with values from rats in which γ-irradiated tumor cells were inoculated. Recent clinical studies, in which plasma PG levels were measured, are in accordance with our results, although 6-keto-$PGF_{1\alpha}$ was found to be increased as well (16,17). In our study 6-keto-$PGF_{1\alpha}$ levels were relatively stable, and were evidently not influenced by tumor progress. Consequently, the TXA_2/PGI_2 ratio was disturbed, a fact which might have had important implications for the homeostasis of platelet/vessel wall interactions (9,18).

Base line levels of TXB_2 and 6-keto-$PGF_{1\alpha}$ are very low, but can easely be influenced by the methodology of blood sampling or small alterations in the hemostatic system (19,20). In this study, for each determination, another rat was taken. This excludes problems, that may arise when blood is taken sequentially from the same rat. However, other factors influencing PG levels, which cannot be controlled easily have also been described, such as hormonal status, dietary composition etc. (21,22). Such variations in PG values, independant of tumor growth, were shown in the present study as well. In the animals in which pieces of either γ-irradiated spleen- or tumor tissue were inoculated, shortly after the inoculation procedure enhanced PG values could be found. The values in the rats in which γ-irradiated spleen cells were inoculated, returned to unoperated control values. However, the rats bearing γ-irradiated tumor tissue showed another peak at 9-13 days in PGE_2

and TXB_2. This unexpected difference between rats, in which either dead spleen or tumor cells were inoculated could only be attributed to some difference in host anti-tumor reaction or to an inflammatory reaction related to the inoculation procedure. This latter is hard to imagine, as there is no reason to presume that inflammatory processes would be different in rats in which either spleen- or tumor tissue was inoculated. Moreover, no visible signs of an inflammatory reaction were noticed. The first explanation, the anti-tumor reaction is also difficult to understand, as the tumor is classified, according to the criteria as described by Prehn and Main as a non immunogenic tumor (23). Still some type of cellular immune response against tumor cells, either dead or alive, may exist (24).

When plasma PGE_2 levels are elevated, the direct consequence of this is a suppression of the production of lymphokines, resulting in a decreased host immune responsiveness, thus providing the tumor a possible means for escape from immunologic rejection (24,25).

Apart from its effect on host immunological responsiveness, high PG levels may also influence metastatic spread (8,26). PGI_2 displays a strong anti-aggregatory action, whereas TXA_2 is a very potent pro-aggregatory agent; the role played by PGE_2 in platelet aggregation is minor and subject to much controversy (27). When in our study 6-keto-$PGF_{1\alpha}$ and TXB_2 values are used to calculate a ratio, as a measure of platelet aggregatory activity, it is clear that pro-aggregatory factors dominate. Platelet function, as determined by us, indeed showed changes. However, the pattern was the inverse picture of what might have been expected on the basis of the determined PG values, i.e. our results showed a decreased aggregatory action instead of an increased one. This makes a direct causal relationship between the observed platelet function and the increase in plasma PG levels not very likely.

To conclude that a relationship exists between high plasma PG values and tumor growth and at the same time altered platelet aggregation, does not seem to be too farfetched. However, it cannot be excluded that concomitant inflammatory processes, more than tumor cells themselves, induce the enhanced PG values. Certainly high plasma PGE_2 values appear to arise predominantly from synthesis by cells infiltrating the tumor. To counteract the increased PG production by administration of PG synthesis inhibitors, and as such possibly improve the rejection of the tumor cells, may be a valuable contribution to anti-tumor therapy. That such a conclusion is not without any foundation, has recently been demonstrated by some investigators (28,29).

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Publication XI

REDUCTIVE EFFECT OF ASPIRIN TREATMENT ON PRIMARY TUMOR GROWTH AND METASTASIS OF IMPLANTED FIBROSARCOMA IN RATS

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Reductive Effect of Aspirin Treatment on Primary Tumor Growth and Metastasis of Implanted Fibrosarcoma in Rats [1,2]

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ABSTRACT—For suppression of primary tumor growth and metastatic spread, aspirin and theophylline, either alone or combined, were given daily to inbred female BN rats after sc implantation of a syngeneic nonimmunogenic tumor. Treatment with 200 mg aspirin/kg (body wt) resulted in a statistically significant regression of tumor growth as well as of the number of metastases in the lungs. Aspirin given in a lower dose (20 mg/kg) did not show significant difference from the vehicle group. Theophylline (75 mg/kg) significantly increased primary tumor growth as well as lung metastases. Inhibition of in vitro platelet aggregation, determined in whole blood taken from non-tumor-bearing animals treated with the same therapeutic regimen, was most pronounced in those groups in which tumor growth and spread were significantly retarded. However, this positive correlation between inhibition of tumor spread and platelet aggregation was not associated with a favorable balance of prostacyclin and thromboxane A_2 in these animals.—JNCI 1986; 76:711-720.

In vivo as well as in vitro synthesis of PG in tumor tissue has been well established (*1-5*). An important finding was that certain PG synthesis products were lower in normal and benign tumor tissue than in malignant tumors of the same organ (*1, 4*). The increases were predominantly in E- and F-type PG; however, in gynecological and prostatic carcinomas, the source appeared to be PGI_2 (*6, 7*). Treatment with aspirin, indomethacin, or other NSAIA sometimes resulted in significant regression of primary tumor growth (*8*) or improvement of the condition of the patients in those cases, when tumor-related complications such as diarrhea in thyroid medullary carcinoma (*9*) or hypercalcemia in malignant breast cancer (*10*) were present. Also, in experimental tumor models, in animals, NSAIA therapy could decrease the mass or the incidence of chemically induced tumors (*11-12*).

Also PG may play a role in the metastatic dissemination of cancer (*13, 14*); therefore, it is not surprising that aspirin therapy proved to be useful in prevention of tumor spread (*15*). Tumor procoagulant activity, possibly the result of TXA_2 activity, was associated with metastasis (*16, 17*). Tumor cell metastasis was enhanced in tumor-bearing mice when the PGI_2-TXA_2 balance was altered in favor of TXA_2 (*18*). However, PGI_2, which acts antagonistically in the control of platelet aggregation, produced a dose-dependent decrease in pulmonary metastatic foci (*19, 20*). An excellent review on the therapeutic implications of manipulating the PGI_2-TXA_2 balance was published by Bunting et al. (*21*).

In cardiovascular surgery much experience has been gathered with aspirin therapy, either alone or in combination with other drugs, concerning its ability to prevent aggregation of thrombocytes (*22*). In these studies sometimes a discrepancy between the effect of low and high doses of aspirin was found (*23*). Moreover, when low doses of aspirin were given in combination with phosphodiesterase inhibitors (these drugs amplify the effect of the increase in cAMP induced by circulating PGI_2), an even stronger antiaggregatory effect was obtained (*24*). These results and the finding that aspirin treatment was not always beneficial in experimental and clinical tumor therapy led us to the present study, in which the hypothesis was tested that aspirin, either alone or in combination with theophylline, could decrease primary tumor growth or the number of metastases in rats.

Moreover, in blood of non-tumor-bearing animals, given the same therapeutic regimen for 3 consecutive days, stable PG end products and platelet aggregation were determined in order to show a possible relationship between these parameters and tumor growth or spread. Non-tumor-bearing animals were willingly chosen so as not to be hindered by a possible interference of tumor PG activity with plasma PG levels and platelet aggregation. As such, the changes in PG levels and platelet aggregation found will exclusively be the result of the therapy given.

MATERIALS AND METHODS

Experimental design.—We randomly divided 65 inbred BN female rats, 100-150 g (body wt), into 6 experimental groups. Group I received aspirin, 20 mg/kg (low

ABBREVIATIONS USED: AA=arachidonic acid; 6-keto-$PGF_{1\alpha}$=6-keto-prostaglandin $F_{1\alpha}$; NSAIA=non-steroidal anti-inflammatory agent(s); PG=prostaglandin(s); PGD_2=prostaglandin D_2; PGE_2=prostaglandin E_2; PGI_2=prostaglandin I_2 (prostacyclin); RIA=radioimmunoassay(s); TXA_2=thromboxane A_2; TXB_2=thromboxane B_2.

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dose) ip; group II, 200 mg/kg of this drug (high dose); group III, theophylline, 75 mg/kg sc; group IV, aspirin at low dose combined with theophylline at 75 mg/kg; group V, aspirin at high dose with theophylline at 75 mg/kg; and, last, group VI, saline, 1 ml (pH 5.2), ip, which was the volume of the vehicle as was given in all experimental groups and which had the same pH as that of the aspirin solutions. The animals received the drugs throughout the whole experiment, which was 15 days, starting on the day after tumor inoculation. The rats were housed in Makrolon cages in groups of 5; food (commercial rat chow) and water were given ad libitum. The animal room was air-conditioned and with a controlled night-dark rhythm.

Determinations of in vitro platelet aggregation and of PG were performed in blood from non-tumor-bearing animals of the same age housed under the same conditions as the animals mentioned above. These animals received the therapeutic regimen for 3 days and were sacrificed for blood sampling at day 4, exactly 24 hours after the last injection.

Tumor inoculation.—The tumor used in this study (BN175) originated from a female animal in one of our longevity studies (*25*), in a rat receiving lifelong a low linoleic acid diet. It was situated in the connective tissue of the pancreas and had metastasized to liver, lungs, and lymph nodes. Slides of the original tumor, stained with hematoxylin and eosin, were identified as a fibrosarcoma by the pathologist of our group (P. Z.) and on the basis of electron microscopy (*see* acknowledgment footnote).

The tumor was best characterized as an undifferentiated mesenchymal tumor; furthermore, on the basis of the fibrous-appearing matrix, the tumor was diagnosed as a fibrosarcoma. After some passages, the tumor progressively became more primitive with most of the cells large with diffuse contours; the nuclei were polymorphic with prominent nucleoli. The cytoplasm was eosinophilic with fairly coarse chromatin granules. Many mitotic figures, some of which were atypical, were present (figs. 1A, 1B).

After inoculation in syngeneic rats, the tumor grew uniformly within a period of 6–8 days to a diameter of approximately 20 mm (fig. 1C). Metastases to lungs, regional lymph nodes, and thymus were present 1–2 weeks after inoculation of the tumor (figs. 1B, 1D). The tumor was stored at −70°C after its 20th passage and was used in experiments after one passage of "starting up" in syngeneic female rates. According to the in vivo transplantation assay described by Prehn and Main (*26*), the tumor should be considered as nonimmunogenic.

In the present study, the tumor was inoculated in pieces of 5–10 mm^3 sc into the right flank of the rat by means of a small incision, which was closed by Autoclips. At day 8, just before surgical removal, the dimensions of the tumor were measured by using a micrometer. Because of the characteristics of the tumor, very soft and not clearly demarcated, removal proved to be cumbersome, especially in those cases when the tumor had invaded the abdominal muscles. All macroscopical visible tumor tissue was carefully removed and bleeding, which was sometimes extensive, was controlled by electrocoagulation. After removal of the tumor, the subcutaneous area was moistened with 4% Formalin in an attempt to prevent local recurrence. The wound was closed with Autoclips.

Fifteen days after inoculation of the tumor, the animals were killed by exsanguination. Local recurrence of the tumor and tumor spread to the axial lymph nodes were scored macroscopically, while the lungs were fixated in Bouin's fixative to enable the counting of the metastases. Lymph node metastases were scored positively when there was evidence of tumor invasion in the right axial region (fig. 1D). Lung metastases were scored semiquantitatively by counting the white nodules on the surface of the left lung and by classifying this count into the score: <11=1, 11–25=2, 26–50=3, 51–100=4, and >100=5.

In vitro platelet aggregation assay.—According to the earlier mentioned protocol, drug treatment was carried out for 3 days in non-tumor-bearing rats. Exactly 24 hours after the last injection, the animals were sacrificed for the determination of platelet aggregation and PG. Under ether anesthesia, after systemically heparinizing the animal (50 IU heparin/rat), blood was drawn by direct aortic puncture. Next, the blood sample (5 ml) was put into a plastic tube containing an additional amount of 25 IU heparin. To eliminate possible effects of endogenous TXA_2 or PGI_2 on the test, the blood samples were left at rest for 30 minutes. Platelets were counted on a TOA Platelet Counter PL100. Platelet function was measured by using a Chronolog-Whole Blood Agrometer (Chronolog Corp., England). We used 1 m*M* AA (PL Biochemical Inc., Milwaukee, WI) as the agent aggregating by virtue of its conversion by platelets into PG endoperoxides. This step is sensitive to nonsteroid inhibitors of the enzyme cyclooxygenase (*27*). In siliconized glass cuvettes, which were kept at 37°C, under constant stirring 0.5 ml saline was added to 0.5 ml whole blood. When a monolayer of platelets was formed on the electrodes, a constant impedance was reached; at that moment, the aggregating reagent was added to the sample. During formation of the hemostatic plug, the changes in electrical resistance (impedance) were recorded on a linear strip chart recorder (Tekman TE 200, 100 mV full scale). Impedance recordings were terminated after 10 minutes.

Determination of PG.—6-Keto-$PGF_{1\alpha}$, TXB_2, the stable breakdown products of PGI_2 and TXA_2, and PGE_2 were determined in blood plasma by means of an RIA, with the use of rabbit antisera (New England Nuclear, Boston, MA). RIA were carried out in plasma taken from the remnant of the blood sample in which the platelet aggregation was studied.

Statistical evaluation.—By use of Student's *t*-test, differences between values of mean tumor size, mean score of lung metastasis, and stable PG end products between experimental groups and the control group were compared. To analyze the differences observed in the meta-

static spread to the axial lymph nodes, we used a chi-square analysis for six random samples. A difference between experimental groups and the vehicle-administered group was considered statistically significant when the calculated P-value was $<.05$.

RESULTS

In group V, the group of animals receiving high doses of aspirin and theophylline, 3 animals died in the 1st week of treatment, apparently due to the toxicity of this combination of drugs. Another rat, in group VI, the control group, died as a result of uncontrolled bleeding after removal of the primary tumor.

From the data given in table 1, it will be clear that the rats in group II and V, receiving the high doses of aspirin either alone or together with theophylline, surpassed in regression of tumor growth and spread. Therapy with low doses of aspirin (group I) did not show any significant improvement in comparison to the result in the vehicle group (group VI). It was rather unexpected that in the rats given theophylline alone, tumor size was increased and metastases to the lungs and regional lymph nodes were more often found. When theophylline was combined with low doses of aspirin, this tumor-promoting effect was for the most part abrogated, indicating an antagonistic effect of these drugs for this particular phenomenon.

Local recurrence of the primary tumor was seen in 35% of the animals; however, as this result was observed among all experimental groups, to the same extent and equally divided, this could have influenced the variation of the results but could not have been responsible for the statistically significant differences found in tumor metastasis (group II vs. group VI).

In table 2, showing the results of the PG measurements, apart from the group of rats treated with theophylline alone, in all experimental groups the values of PG end products tend to be decreased. Although a limited number of animals were available in each experimental group, statistically significant differences could be shown when treatment groups were compared with the vehicle-administered group. Most pronounced differences in the level of PG end products were established in the group of rats treated with 200 mg aspirin/kg and theophylline. Text-figure 1 shows the AA-induced platelet aggregation pattern in blood from these rats. Rats from group V (showing the strongest inhibition of PG synthesis) also showed a marked inhibition of platelet aggregation. Furthermore, it was striking that in group III, in which the rats received therapy with theophylline alone, 6-keto-$PGF_{1\alpha}$ and TXB_2 were slightly increased, whereas in the platelet aggregation a slight hyperaggregation seemed to be present.

Changes in either PG levels or platelet aggregation correlated with changes in tumor growth and metastasis as well. Whereas treatment with 200 mg aspirin/kg and theophylline (group V) was superior in blocking platelet aggregation and PG synthesis, this group was also the group in which the effect of tumor growth and spread was most prominent. In group III, the same correlation was found in an inverse way; a slight hyperaggregability and increased 6-keto-$PGF_{1\alpha}$ and TXB_2 values were accompanied with enhanced tumor growth and lung metastasis. However, no such correlation was found between the effect of low-dose aspirin on platelet aggregation and the effect of this treatment on tumor growth. Whereas in platelet aggregation almost no difference could be detected between high- and low-dose aspirin, with regard to tumor growth and metastasis, the animals treated with low dose of aspirin showed no significant difference from the vehicle group. Also, the rats from group II, superior in the reduction of tumor growth and metastasis, do not have a favorable effect on PG synthesis and aggregation.

DISCUSSION

There is mounting evidence that treatment with NSAIA, like aspirin, is able to reduce primary tumor growth (*12, 28*) or the number of tumor metastases (*13*). These favorable effects of aspirin-like drugs are supposed to be the result of blocking the enzyme cyclooxygenase, which is necessary for the metabolism of numerous PG. As PG play a role in cell metabolism and are found in large quantities in cultures of tumor cells as

TABLE 1.—*Effect of aspirin and theophylline treatment on tumor growth and spread*[a]

Tumor status	Group I, $n=10$	Group II, $n=10$	Group III, $n=10$	Group IV, $n=10$	Group V, $n=7$	Group VI, $n=14$
Tumor size, mm, mean length plus width	19.8 (0.6)	17.1[b] (0.7)	21.1 (0.5)	20.7 (0.7)	16.9[b] (1.3)	19.7 (0.6)
Involvement right axial lymph node, +/−[c]	7/3	2/8[b]	9/1	9/1	4/3	8/6
Lung metastases, mean score[d]	2.3 (0.4)	1.2[b] (0.1)	4.0 (0.4)	3.4 (0.3)	2.1 (0.6)	3.1 (0.4)

[a] Group I, 20 mg aspirin/kg (body wt) given ip; group II, 200 mg aspirin/kg ip; group III, 75 mg theophylline/kg sc; group IV, 20 mg aspirin/kg ip and 75 mg theophylline/kg sc; group V, 200 mg aspirin/kg ip and 75 mg theophylline/kg sc; group VI, 1 ml vehicle ip. Treatment was started immediately after inoculation of the tumor. Values given *in parentheses:* SE

[b] Statistically significant from the vehicle-administered group, $P<.05$.

[c] + = involvement; − = no involvement.

[d] Mean score of the number of lung metastases is determined by calculating the mean of individuals' scores on the scale: <11=1, 11–25=2, 26–50=3, 51–100=4, and >100=5, as was established by the counting of white metastatic nodules on the surface of the left lung.

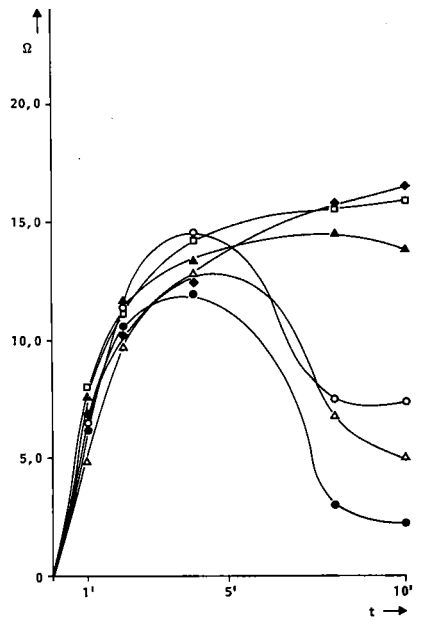
TABLE 2.—*Effect of aspirin and theophylline treatment on PG synthesis in non-tumor-bearing rats*[a]

End products	Group I, $n=3$	Group II, $n=3$	Group III, $n=3$	Group IV, $n=3$	Group V, $n=3$	Group VI, $n=3$
6-Keto-$PGF_{1\alpha}$	75.3[b]	98.0[b]	143.3	77.7[b]	72.3[b]	136.0
	(6.6)	(13.6)	(18.2)	(21.1)	(8.4)	(14.0)
TXB_2	121.3	145.5	156.0	74.7[b]	86.0[b]	148.7
	(18.7)	(43.3)	(18.9)	(31.2)	(7.0)	(33.4)
PGE_2	129.3[b]	165.3[b]	161.3	172.0	97.3[b]	220.0
	(9.6)	(21.0)	(38.3)	(42.4)	(7.4)	(18.0)

[a] Group I, 20 mg aspirin/kg (body wt) given ip; group II, 200 mg aspirin/kg ip; group III, 75 mg theophylline/kg sc; group IV, 20 mg aspirin/kg ip and 75 mg theophylline/kg sc; group V, 200 mg aspirin/kg ip and 75 mg theophylline/kg sc; group VI, vehicle 1 ml ip. Determinations of RIA were carried out exactly 24 hr after the last injection of the drugs (3 consecutive days). Values given in pg/ml plasma *in parentheses*, SE.

[b] Statistically significant from the vehicle group, $P<.05$.

metabolic degradation products (*1*), the observed effect of aspirin in in vitro studies can be the result of a direct action of aspirin on tumor cell metabolism (*29*). However, as aspirin also may play a more central role, either via PG or other pathways, on the immune system, other mechanisms may be involved as well (*30, 31*).



TEXT-FIGURE 1.—Effect of aspirin and theophylline treatment on AA-induced platelet aggregation in non-tumor-bearing rats. Determination of platelet aggregation was carried out exactly 24 hr after the last injection of the drugs (3 consecutive days). △, group I, 20 mg aspirin/kg (body wt) given ip; ○, group II, 200 mg aspirin/kg, ip; ◆, group III, 75 mg theophylline/kg, sc; ▲, group IV, 20 mg aspirin/kg, ip, and 75 mg theophylline/kg, sc; ●, group V, 200 mg aspirin/kg, ip, and 75 mg theophylline/kg, sc; □, group VI, vehicle. On the *x*-axis, time (t) is plotted in min (′); on the *y*-axis, platelet aggregation is given as the impedance (Ω); *commas before zeros* mean *decimal points*. Three rats were studied at each time point.

One of the possible mechanisms by which cyclooxygenase inhibitors are able to act on the prevention of tumor spread is also thought to be a more central one. The antiaggregatory action of aspirin-like drugs would prevent tumor cells sticking to the endothelial wall of the circulatory or lymphatic system and, as such, reduce the formation of new tumors (*14*). In this respect, it is also important to consider the direct aggregatory action of tumor cells on platelets (by means of release of TXA_2?) and on vasoconstriction, as such creating favorable conditions for subsequent evolution of tumor emboli (*16*). Considering these phenomena, it is surprising that so little attention has been paid to the dosages of the therapeutic regimen. Because to create favorable circumstances it is necessasry to obtain unaltered or, even better, an enhanced synthesis of PGI_2 and a decreased TXA_2 synthesis, in this respect, it seems to be important to give therapy with low doses of aspirin.

In studies on the antiaggregatory activity of aspirin on platelets, Moncada and Vane (*22*) pointed out that high and low doses of aspirin had different effects on the formation of PG. They showed that aspirin in high doses blocked the cyclooxygenase pathway, resulting in an almost complete blockade of PGI_2 and TXA_2 synthesis. However, low dose of aspirin, in humans 1 mg/kg, decreased TXA_2 values substantially, whereas PGI_2 synthesis was left intact; these original findings were confirmed by others as well (*23, 32*). According to the above-mentioned studies, therapy with low doses of aspirin alone or possibly combined with phosphodiesterase inhibitors would be the best choice to achieve those conditions with the best potentials to reduce or prevent tumor spread.

In the present study, there is not much evidence for favorable antiaggregatory conditions when therapy with 20 mg aspirin/kg either alone or combined with theophylline is given to non-tumor-bearing animals. Although the measurements of PG synthesis and platelet aggregation detected a reduced function, this effect was certainly not restricted to the group of animals receiving the therapy with low doses of aspirin in combination with theophylline. And in comparison with all other groups, the PGI_2-TXA_2 ratio was even most unfavorable in this particular group. However, we have to realize that the determinations were carried out 24 hours after

the last injection of the drugs and therefore show more or less the steady state level of PG synthesis and platelet function. It is possible that early after the injection of the drugs, other levels of PG synthesis or aggregation would have been found, more in accordance with the data stated in the literature.

The method of determining platelet aggregation as has been used in this study needs some further comment as well. Measuring platelet aggregation in whole blood is a rather new method, but in several studies this technique has proved to be very worthwhile in showing changes in platelet function (*26*). Although basically the method of measuring platelet aggregation electronically is different from the classical way of determining platelet aggregation by means of optical changes in platelet-rich plasma, comparison of the patterns obtained by the two different methods is not so difficult. Apart from the simplicity of the method, measuring in full blood has the advantage that, especially for those interested in the involvement of PG synthesis on platelet aggregation, the contribution of the erythrocytes to the process of aggregation is still present.

Although Honn (*20*) found excellent results with treatment with PGI_2, thromboxane synthetase inhibitors, and a TXA_2 receptor blocker in order to decrease metastasis, in numerous other studies such a therapy to create favorable conditions in the prevention of tumor metastases according to the PGI_2-TXA_2 balance theory did not give any improvement and in other studies even an increase in the number of metastases was noted (*33–35*). Because the less favorable results were obtained in models in which spontaneous dissemination of tumor cells were studied, it is possible that modulating the vascular PGI_2-TXA_2 system acts beneficially only in systems in which "tumor take" is established, which is the case in the protocol as has been used by Honn (*20*) when tumor cells were injected iv shortly after the therapeutic regimen was given.

In the present study, after therapy with 200 mg aspirin/kg, not only was the number of metastases most significantly reduced but also a reduction of the primary tumor was obtained. To a certain extent the beneficial effect on metastatic spread could be thought to be related to the obtained reduction of the primary tumor, as one may expect that there is a linear correlation between tumor size and the number of metastases. However, a follow-up of this study, in which a different experimental design was used—amputation of the leg, in which the tumor had been inoculated and therapy started after the removal of the tumor—the results showed the same pattern: The group of animals receiving 200 mg aspirin/kg was the group with less tumor metastases in the lungs (*35*). Amputation of the leg was performed in this study to prevent the unfavorable effect of recurrence of the primary tumor, which is a possible weakness of the experimental model used.

It becomes increasingly obvious that, other than the earlier discussed PGI_2 and TXA_2, other PG also may play an important role in tumor growth and metastases. In a number of tumors, high amounts of PGE_2 were found; moreover, a relationship between the level of PGE_2 and tumor progression was noted (*1, 36*). These findings implicate that certainly for such tumors, like in our study, a more complete blockade of the cyclooxygenase pathway is needed to obtain an alteration of the tumor progression.

Although, so far, many investigators found favorable effects of NSAIA treatment on tumor growth, this was certainly not always the case. In some other studies, no improvement or even a deterioration of the tumor process was found (*37*). For instance Stringfellow and Fitzpatrick (*38*) observed that the metastatic pattern of 2 melanoma cell lines were dependent on PGD_2 synthesis; i.e., the tumor with low synthesis capacity of PGD_2 was the one with a high number of metastases, whereas the one with the low number of metastases could significantly synthesize higher amounts of PGD_2 (*39*). When indomethacin was given to tumor-bearing mice, the 2 tumors acted identically; the metastatic rate was increased, particularly in the tumor cell line with the least metastatic potentials. This illustrates once more the complexity of the relation between PG synthesis and tumor spread. Nevertheless, clinically, in many cases therapy with PG or their synthesis inhibitors seems to be potentially beneficial in the control of tumor growth and spread. Even manipulating PG synthesis by increasing or decreasing the amount of linoleic acid in the diet might be beneficial in the control of tumor growth and metastasis (*40, 41*). Observed alterations in tumor growth or tumor incidence in experimental animals on high linoleic acid diets are often attributed to the polyunsaturated fatty acid content of the diet (*25, 42, 43*). Indomethacin was capable of blocking the stimulatory effect of high dietary fat in mammary tumorigenesis (*44*).

However, either one of these therapies (or a combination of one of the therapies together with classical therapies) needs a background of detailed knowledge concerning the PG synthesis of the tumor involved. Furthermore, it is challenging to investigate whether the values obtained by measuring the platelet aggregation of tumor-bearing individuals can be used as a parameter for monitoring the tumor progression.

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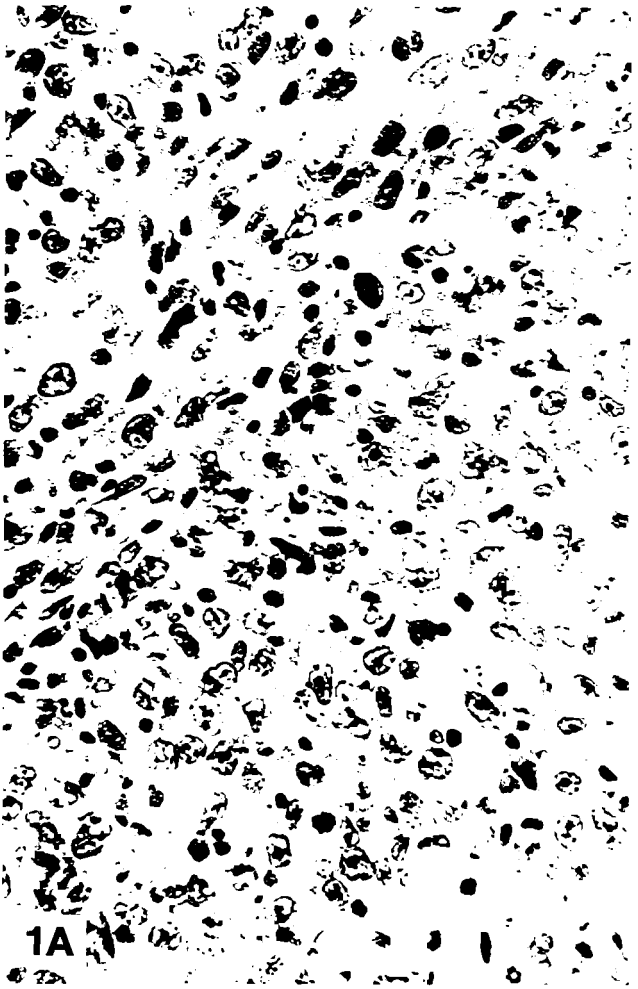

FIGURE 1A.—Primary tumor growth of the inoculated fibrosarcoma demonstrates large pale cells with polymorphic prominent nucleoli. Fibrous-appearing matrix and many mitoses can be seen. Hematoxylin and eosin. × 500 (original magnification, × 380)

FIGURE 1B.—Tumor metastases in the lung showing the same characteristics as the primary tumor. Hematoxylin and eosin. × 80 (original magnification, × 60)

FIGURE 1C.—Macroscopic appearance of the tumor inoculum in the right flank at day 8.

FIGURE 1D.—Lymph node metastases in lumbal (I) and inguinal (II) nodes.

In the midst of the word he was trying to say,
  In the midst of his laughter and glee,
He had softly and suddenly vanished away .....
  For the Snark **was** a Boojum, you see.

THE END

From "The Hunting of the snark", by Lewis Carroll