TUBE LEUKOCYTE ADHERENCE INHIBITION ASSAY

The assessment of tumor immunity in cancer patients and in rats

DE LEUKOCYTEN-ADHERENTIE-REMMINGS TEST

Bepaling van tumor immuniteit bij kanker patiënten en ratten

PROEFSCHRIFT

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To my Parents
For Ria

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 Brief history of tumor immunology

The history of tumor immunology can be considered to have evolved over three eras. The first era began towards the end of the 19th century when it was observed that cancer could sometimes be transmitted from one animal to another by transplantation of tumor tissue (1,2). Subsequently, it was observed that while some tumors like Ehrlich mouse carcinoma and Jensen rat carcinoma grew progressively resulting in the eventual death of the host, many other tumors grew only for a brief period and then regressed completely. The investigations of Ehrlich (3,4) demonstrated that mice with an already established tumor transplant often failed to produce a further tumor when reinoculated with tumor cells, suggesting that the tumors possessed particular antigens not occurring on normal cells. These observations were confirmed and extended by other investigators in several laboratories, and sometimes, using different terminology for the tumor-associated antigens (TAA). However, many false conclusions on the same observations that were made in various laboratories could be attributed to lack of genetically homogeneous strains of laboratory animals. One of the most valuable contributions of this era came from Murphy in 1926 on the role lymphocytes played in resistance to tissue grafts, and malignant diseases (5).

The second era began with the development of inbred strains of laboratory animals by C.C. Little at Bar Harbour. This initiated a tremendous surge in immunogenetic investigations by several prominent investigators (6). As a result, the genetic basis of histocompatibility was firmly established, specific immunological tolerance and the graft-versus-host (GVH) reaction were discovered and a vaste amount of data on the mechanisms of allograft rejection, methods of immunosuppression, and biochemistry of transplantation antigens was further accumulated.

Since considerable discrepancies exist in the correct terminology in the literature, the following definitions are introduced. Tumor-associated antigens (TAA) are defined as determinants which are present on tumor cells but are either totally absent or present to a limited extent only on normal cells. Tumor-specific antigens are defined as determinants that are present on a spontaneous tumor of a particular type in an individual and are totally absent on the cells of the same type of tumor in another individual and on normal cells.

The discovery of TAA in 1943 by Gross marked the birth of the third era. It was firmly established that a variety of tumors possessed TAA (7). Furthermore, TAA

of some tumors included a subset of cell-surface antigens that were termed tumor-associated transplantation antigens (TATA) which could evoke an immune response in other animals syngeneic with the animal in which the tumor originated (7). It was also established that autotransplants of some tumors induced resistance in the animal in which the tumor originated. Klein and Oettgen (8) reported that autotransplants of some tumors encountered a stronger barrier than similar tumor transplants in untreated syngeneic recipients suggesting that such tumors possessed the above defined tumor-specific antigens with a rejection-inducing potential in the autochthonous host.

1.1.1 *Tumor-associated antigens (TAA)*

The existence of TAA in most animal tumor systems has been confirmed on the basis of the rejection of experimentally induced tumors inoculated into previously immunized syngeneic recipients (9,10). Oncofetal antigens (OFA) are found in fetal and malignant tissues, but are totally absent or only present in very small amounts in normal adult tissues (11). These normal antigens in the foetus are thought to be repressed as the process of intrauterine development proceeds towards birth and then de-repressed during the malignant transformation process. The existence of OFA supports the idea that cancer represents a de-differentiation to a more primitive cell type. The relationship between neoplasms, specific tumor antigens and fetal antigens is not clear. Carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) are the most extensively investigated OFA (12,13). The most apparent importance of these antigens is their ability to serve as markers for various cancers. Both CEA and AFP are neither disease specific nor do they have any clear correlation with the prognosis for patients as a whole, but they do have some prognostic value for individual patients. Little information exists as to whether the OFA are organ specific. In recent studies, Thomson has reported that fetal organs expressed the organ specific neoantigens (OSN) at about 13-19 weeks (14).

However, todate, no true tumor-specific antigen or TAA has ever been isolated from spontaneous tumors in contrast to numerous virus induced tumors. This thesis is confined to spontaneous tumors only and therefore tumor-specific antigens and TAA will be discussed in this context. Comparable direct evidence for the existence of TAA in human tumors is also not available.

The existence of TAA in many human tumors is suggested by the accumulation of lymphocytes and plasma cells in the tumor stroma, the presence of immunoglobulin on tumor cells, and histological changes characteristic of an immunological reaction in the regional lymph nodes. The presence of TAA has been indirectly established by the demonstration of antigenic differences between tumor cells and normal cells on xenogeneic immunization, and in vitro evidence of immunity to tumor cells or extracts mediated by the patient's serum or lymphocytes. Recently it has also been proposed that TAA displayed by some tumor cells may be closely related to normally occurring major histocompatibility complex (MHC) coded structures (15).

The principal data supporting the concept that human tumors express TAA similar to those detected on experimental tumors has been obtained from in vitro assays of cell-mediated and humoral antibody responses to tumor cells (16). However, the validity of the in vitro approaches for assessing whether tumors do indeed express neoantigens capable of host recognition has been questioned on the basis of a complete lack of histologic type-specific reactivity by cancer patients (17,18,19). Recently, direct evidence confirming the existence of TAA in human tumors has been obtained by the successful development and use of monoclonal antibodies which were capable of demonstrating antigens that were gained in malignant transformation (20-23).

TAA represent a wide spectrum of antigens with different implications in terms of immunogenicity in the autochthonous host, uniformity of expression and specificity. For effective immunotherapy, it is desirable to have an immunogenic antigen which is uniformly found in all tumors of a given histological type, is not found in benign disease and is located on the surface of all tumor cells. Unfortunately, TAA are rarely ideal. An antigen that is immunogenic in one patient with cancer, may not be immunogenic in another because of genetic variations in immune-responsiveness and what is considered "self". Since TAA of some experimentally induced animal tumors appear to be weakly immunogenic, the anti-tumor immune response evoked by the tumor host would be correspondingly weak (24). Nevertheless, these immune responses are more tumor-directed than those which may be evoked by the immunization of xenogeneic animals.

The host's anti-tumor immune response has been used to identify TAA (23,25,26,27). In vitro assays of anti-tumor immunity of the human host have until recently, been found unreliable to monitor the isolation of the antigen(s) involved in the response. Furthermore, studies dealing with the detection of human tumor antigens by the immunization of xenogeneic animals were fraught with problems (28).

1.2 **Methods for the detection of tumor immunity**

1.2.1 *In vitro tests for humoral immunity*

In vitro tests for humoral immunity in tumor-bearing animals or cancer patients include complement fixation, various tests based on the precipitin reaction, lysis of tumor cells in the presence of complement (10), immunofluorescence on fixed cells, membrane immunofluorescence (29), the indirect radioactivity labeled antibody technique (30), immune adherence (31), the mixed antiglobulin reaction (32) and the related mixed hemadsorption test (33) and inhibition of tumor cell mobility (34). The standard complement (C) fixation test and immunofluorescence with fixed cells detect antigens within as well as on the surface of the cell. The modified C1a fixation and transfer test (35) and membrane immunofluorescence, however, detect only cell surface antigens.

The reactivity of sera from cancer patients with intracellular antigen of their own

or a histologically similar tumor, or cell lines which are derived from such tumors has been demonstrated by complement fixation (36), precipitin reaction with antigenic tumor extracts (37) and immunofluorescence on fixed cells (38) in Burkitt's lymphoma, leukemias, sarcomas, melanoma and carcinomas of the kidney, colon, skin, nasopharynx and cervix (36,37,38). On the whole, using these techniques, considerable cross-reactivity between tumors of the same histological type was observed although there was also some evidence for some specific antigens in individual human colon carcinomas (39). The literature concerning cross-reactivity between TAA from tumors of different origins is complex and confusing.

1.2.2 *In vitro tests for cell-mediated immunity*

1.2.2.1 Antibody-dependent cell-mediated cytotoxicity (ADCC)

ADCC is a test in which a variety of target cells can be lysed by cells from non-immunized donors in the presence of specific antiserum or immunoglobulin G (IgG) antibody raised in xenogeneic and allogeneic animals in the absence of complement. This phenomenon has also been demonstrated with antiserum from mice immunized with a syngeneic tumor and human effector cell (40). Various types of cells (K cells, monocytes, macrophages and polymorphonuclear leukocytes) bearing Fc receptors that can bind IgG function as effectors in ADCC (41). Subclasses of T and B cells also mediate ADCC via immunoglobulin M (IgM) Fc receptors that bind IgM-antigen complexes (42). Lymphocytes with T-cell markers can also co-operate with IgG antibodies in the lysis of human tumor cells in vitro (43).

1.2.2.2 Lymphocyte-mediated cytotoxicity

Lymphocyte-mediated cytotoxicity can be assessed using three types of assays:
a. *The colony inhibition (CI) assay:*
This assay was developed by Hellström in 1967 (44). In this assay a comparison is made between the number of colonies which develop in tissue culture plates seeded with tumor cells as target and lymphocytes as effectors and the number of colonies which develop on plates seeded with tumor cells only. In the cloning inhibition test, a modified version of CI, the target cells and the effector cells are preincubated together in bulk before being seeded into microplates (45).

b. *Long-term (20-72 hours) cytotoxicity assays*
Several different techniques have been developed and discussed in details elsewhere (46). The microcytotoxicity assay of Takasugi and Klein (47) is based on the enumeration of adherent cells remaining in the wells of a microtest plate at the end of the incubation period with effector lymphocytes. The enumeration can be done manually after Giemsa or some other staining or it can also be done electronically (48). Other methods are based on labeling the target cells with ^{3}H-thymidine (49),

^{3}H-uridine (50), ^{125}I-iododeoxy-uridine (^{125}I UDR) (51) or ^{3}H-proline (45), and the measurement of the amount of radioactivity in the supernatant. The assays are usually set up with various effector to target cell ratios, depending on the type of the assay.

c. *Short-term (4-8 hours) cytotoxicity assays*
These assays are based on the release of ^{51}Chromium (^{51}Cr) from labeled target cells. This method of labeling is unsatisfactory for long-term assays since, there is a high amount of spontaneous leakage of the isotope from the tumor cells incubated in the absence of effector cells.

Clinical results
A large number of patients with a wide range of tumors including carcinomas of the ovary, thyroid, skin, buccal cavity, colon, breast and testis have been found to give frequent positive reactions when their lymphocytes were tested in CI or long-term cytotoxic assays with their own tumor, or with allogeneic tumors of the same histological type including cell lines (52). The results were similar when both tests were used simultaneously. Lymphocytes which are cytotoxic for autochthonous tumors have usually been found to be cytotoxic also for allogeneic tumors of the same histological type, but not for other tumors (45,52). While this observation clearly suggests the existence of TAA that are found on all tumors of a particular type (group-specific TAA), the existence of some antigens specific for individual tumors from individual patients is not excluded, and there is evidence that these are possessed by some tumors. Some of the antigens found on tumors may also occur in benign lesions; thus cross-reactivity has been reported between carcinoma and benign hyperthrophy of the prostate (53) and carcinoma of the breast and benign fibrocystic disease (54). In some instances a patient's lymphocytes cease to be reactive in cytotoxic tests when his/her tumor becomes widely disseminated (55).

Short-term cytotoxic assays have performed rather poorly. Inconsistent results with an increased non-specific cytotoxicity and a poor correlation with long-term assays has been frequently observed (56). However, since most of the short-term assays have been performed with allogeneic tumor cell lines, the results using autochthonous tumor material could well be of better consistency.

1.2.2.3 Migration inhibition assays

Migration inhibition assays are based on the observation that when sensitized T cells come in contact with the sensitizing antigen, lymphokines are released by the T-cells which inhibit the migration of leukocytes and macrophages. Two types of assays have been developed: The direct migration inhibition assay (57) and the indirect migration inhibition assay (58).

In the direct assay, the migration of blood leukocytes is measured in the presence or absence of antigen; if sensitized T cells are present, the migration inhibition

factor (MIF) they release, inhibits the migration of other leukocytes in the population which serve as indicator.

In the indirect assay, lymphocytes are incubated with or without antigen, and the capacity of the culture supernatants to inhibit the migration of guinea pig peritoneal exudate cells is measured.

The main limitation of the direct test is the determination of optimum concentration of tumor extract at which the concentration of toxic substances in the extract is minimal (59). Despite this technical difficulty, evidence of sensitization of lymphocytes to TAA in patients with carcinoma of the breast (60), colon (61), malignant melanoma (62) and other tumors (63) was obtained using direct tests in which antigenic extracts of autochthonous tumors and allogeneic tumors of the same histological type were used. In the majority of these studies, there was evidence of cross-reactivity with allogeneic tumors of the same type.

The indirect assay would appear to be less prone to errors and shows a good correlation with skin hypersensitivity tests using soluble protein antigens, but surprisingly has been less widely used than the direct assay. In a small study, positive results with autochthonous tumor antigens were observed in 4 of 7 patients using the indirect assay (64). However, in the same study, no definite evidence of sensitivity to carcinoembryonic antigen in nine patients with colon carcinoma was obtained using the indirect assay.

A modification of the migration inhibition assay was devised by Field and his collegues (65). In this version, human blood lymphocytes were mixed with guinea pig macrophages, with or without the addition of antigenic material derived from human brain, sciatic nerve or any of a variety of tumors. The rate of migration of individual marophages under the influence of an electric field was then measured with a cytopherometer. After extensive investigations these authors came to the conclusion that lymphocytes from patients with a wide variety of tumors were sensitized to a basic histone-like protein which can be extracted from human brain and peripheral nervous tissue. It was suggested that this test could provide a suitable diagnostic tool of malignancy. Todate this work has neither been confirmed nor refuted by other independent investigators.

In 1972, Halliday and Miller reported on the phenomenon known as leukocyte adherence inhibition (LAI) in tumor bearing mice (66).

1.3 **Leukocyte Adherence Inhibition (LAI) assays**

1.3.1 *A brief review*

The Leukocyte Adherence Inhibition (LAI) assay has evolved directly from the macrophage migration inhibition (MMI) assay which was used with some success by Halliday and coworkers (67,68,69). The MMI test is based on the reactivity of specific antigen with immune lymphocytes which then release substance(s) capable of inhibiting the migration of macrophages. However, since this assay was both tedious and time consuming, the idea that perhaps, the adherence of macrophages

to a solid surface during a brief incubation period might be analogous to their migration during longer periods as an indicator of lymphocyte activity, was conceived and the leukocyte adherence inhibition (LAI) test was born. This test is based on the phenomenon that sensitized leukocytes, not necessarily containing macrophages, lose their ability to adhere to glass or plastic surfaces when exposed to the sensitizing antigens in vitro. The first results utilizing this assay to demonstrate specific cell-mediated immunity and serum blocking factors in inbred mice with transplanted chemically induced tumors were published by Halliday and Miller in 1972 (66).

Since the first introduction of the original hemocytometer LAI (66), several modified versions of the assay have been developed during the last decade aiming at a simple, automated and reproducible method. All existing variations of the LAI assay fall into one of the three general categories; hemocytometer LAI (66), microplate LAI (70) and tube LAI (71) methods. In all the three categories, adherence has been studied in relation to polystyrene and glass surfaces on which interaction occurs. Both these materials have been used in various forms such as glass hemocytometers (66), glass tubes (72), 96-well polystyrene tissue culture microtest plates (73,74) and small-sized polystyrene tissue culture microtest plates (70). For an accurate estimation of adherent and nonadherent cells, machine aided techniques which utilize an automated light microscope (75,76,77) or a coulter counter (74,78) have also been introduced. A radio-isotopic method in which radioactive chromium (^{51}Cr) prelabeled cells are used and radioactivity is measured instead of the enumeration of cells has also been successfully developed and used (73,79). Recently, a micro-glass-tube LAI assay in which the microscopic enumeration of the specific monoclonal antibody reagents bound to the adherent cells in glass tubes has been developed and successfuly used (27,80).

The individual merits of the three general categories of the LAI assays have also been the subject of an international workshop held in 1978 (81). Although, the three categories of LAI assays were found to have different mechanisms, the general consensus of the workshop was that the LAI was a promising aid for monitoring cell mediated immunity (CMI) in cancer. In comparison to the other methods for the detection of CMI (43,44,45,57,58), the LAI assays were found not only to be simpler and faster, but could also detect serum blocking factors (antigens, antibodies and/or immune complexes). In addition, they also had the essential properties of specificity, reproducibility and correlation with the stage of disease for minitoring cancer patients. An extensive review on the development of various LAI techniques, their mechanisms and their applications in man an domestic animals has been published by Thomson (82). The general consensus of this review is that LAI assay is a valuable tool to study tumor immunity in man and animals.

1.3.2 *General methodology*

The LAI test is based on the phenomenon of decreased adherence of leukocytes to glass or plastic surface when exposed to tumor associated antigens against which

the leukocyte donors (tumor-bearers) have been sensitized. The test hinges on the existence of organ specific TAA. The general source of these antigens is crude extracts that are prepared by homogenization or 3M potassium chloride (KCl) treatment of allogeneic tumor material. Alternative sources of these antigens include serum, urine and spent tissue culture media of tumor cell lines.

1.3.3 *Hemocytometer LAI assay*

The LAI-reaction that is observed in this technique is based on the production of a soluble mediator, a lymphokine, called the Leukocyte Adherence Inhibition Factor (LAIF). The action of LAIF on adherence can be detected in a one-stage, *direct assay* or in a two-stage, *indirect assay.*

Direct assay

The hemocytometer LAI as originally described by Halliday and Miller (66) consists of reaction mixtures containing tumor extract and leukocytes suspended in medium supplemented with normal serum in plastic tubes. The mixtures are incubated for 30 minutes at 37°C with intermittent shaking to prevent the cells from adhering. The mixtures are then transferred to hemocytometer chambers and incubated for an additional one hour at 37°C to permit adherence. At the end of this period the cells are counted. This is followed by a gentle rinsing procedure which appears to be very criticle and requires some dexterity and routine. The adherent cells are then counted.

Indirect assay

An active supernatant is prepared by preincubation of specifically reactive leukocytes with tumor extract. The LAIF in the supernatant is then detected by its action on normal indicator cells in a second stage. An alternative method of LAIF preparation is to "pulse" the leukocytes with tumor extract for two hours. After centrifugation, the supernatant is discarded and fresh medium is added and the cells are incubated for a further 24 hours. The resulting supernatant is harvested and can be stored frozen. This method has the advantage of having no tumor extract present in the supernatants at the time of testing with indicator cells. The non-specific effect of the tumor extract on adherence is thus eliminated.

The indirect assay has several advantages. LAIF can be quantitated and several supernatants can be evaluated using a single batch of indicator cells. The LAIF containing supernatants can be stored frozen and used when required.

Putative mechanisms

Investigations into the putative mechanisms of the hemocytometer assay have been conducted using several kinds of defined antigens as well as tumor extracts. Species that were examined include mice, rats, guinea pigs and humans. LAIF as a soluble mediator in the hemocytometer LAI assay both in experimental animals and man has been confirmed by several independent investigators (83-90). In all these

studies, the presence of T-lymphocytes with or without macrophages as accessory cells for the generation of LAIF was found to be imperative. In addition, Dunn and Halliday (88) using spleen cells of sensitized mice showed for the first time that two populations of B cells were also involved; one population that specifically produced LAIF while the second, in the presence of specific antigen, suppressed the LAI-reaction. They also obtained evidence for a suppressor T cell that regulated LAIF production by B cells in mice. These findings have not been confirmed yet.

Todate there are no reports of the cell types involved in LAIF production with human tumor antigens, although LAI response to these antigens is mediated by a soluble factor. Noonan et al. (91) have demonstrated an adherence stimulating factor in response to tumor antigens, and it is possible that the LAI observed is the net result of the action of both LAIF and the adherence stimulating factor under appropriate conditions. Murine blood leukocytes from tumor-sensitized animals reacting with tumor extracts resulted in a well-defined indirect LAI-reactions similar to those observed with murine peritoneal cells (86).

The adherence inhibiting activity of LAIF also seems to be very aselective, effecting all major types of allogeneic leukocytes (92), syngeneic leukocytes and xenogeneic leukocytes (39).

An important feature of serum inclusion in this assay enables the investigation of blocking and other serum factors. Blocking factors (BF) are specific inhibitors of in vitro reactions of cell mediated immunity deteted in sera of animals and humans with cancer (94). It has been demonstrated in numerous studies that the sera of tumor-bearers blocked the LAI specifically (66,85,95,96,97), whereas the sera of ex-tumor-bearers often show unblocking properties. BF are believed to be circulating antigen-antibody complexes (98,99) or excess shed tumor antigens in the circulation which block the leukocyte-antigen interaction (100,101). Sera from mice or man, obtained soon after tumor removal or regression, contain substances that inhibit BF. These have been suggested to be antibodies to BF (102).

Since the presence of BF or unblocking serum factors may directly reflect the tumor state and prognosis, their detection could play a significant role in the immunological monitoring of treated cancer patients for recurrence or for residual disease. However, the hemocytometer LAI assay does not discriminate between the early or late stage of disease or recurrent disease (103). Specific leukocyte activity was observed in all stages of tumor growth using this assay. These findings contradict those obtained using the tube LAI, where leukocytes from patients with disseminated disease were constantly non-reactive (101).

1.3.4 *Microplate LAI assay*

The microplate LAI assay was originally developed by Holt and coworkers in 1974 (70,104). Similar to the hemocytometer LAI assay, this assay is also based on the release of a soluble mediator(s) from sensitized lymphocytes upon contact with specific antigen.

The microplate assay is performed in microtest tissue culture plates. The test is set up in triplicate wells. Each well contains 1 μl of tumor extract and 5×10^3 viable leukocytes in 10 μl of RPMI 1640 medium supplemented with 10% serum, usually fetal calf serum (FCS) or serum syngeneic to the experimental animal under test. The plates are then incubated at 37°C in a humidified CO_2 incubator for 2 hours, at the end of which they are inverted for 15 minutes to allow the sedimentation of the bulk of nonadherent cells. The plates are then carefully rinsed, and the adherent cells at the bottom of the well are fixed with methanol, stained with 0.1% toludine blue and counted.

Putative mechanisms

Variables such as the incubation time, the rinsing procedures and the counting devices that were used in the microplate LAI assay (73,75,105,106) may play a considerable role in the various putative mechanisms of this assay. Holt and coworkers (104), using bacterial and viral antigens demonstrated the obligatory role of T-lymphocytes which released LAIF. Creemers (107) showed by means of an indirect LAI assay, using a murine mammary tumor virus model, the involvement of T-lymphocytes and three apparently distinct soluble mediators that were sequentially produced. Russo et al. (73) and Goldrosen et al. (105) using a murine colon adenocarcinoma (MCA-38) model demonstrated that in this system the mediator was an immunoglobulin that was released from sensitized B cells. This immunoglobulin then inhibited the adherence of monocytes. This putative mechanism is similar to that of the tube LAI (72). These discrepancies in the postulated mechanism(s) of the microplate assay were investigated by Holt et al. (108) who pointed out that Goldrosen et al. (105) assayed a more strongly adherent population and also cells of a greater average diameter than those used by Holt et al. (104) and Creemers (107). The confirmation that both T and B lymphocytes were essential in the microplate assay was reported by Mortensen and Elson (106).

The microplate LAI assay was found to be more sensitive than the cytotoxicity assays for the detection of blocking factors (109,110) and compared favourably in most respects with other established tests for the assessment of host cell reactivity to a variety of tumor, bacterial and viral antigens in mouse and man (111). Leveson et al. (112) and Mortensen (113) also observed that the leukocytes from patients with large tumor burdens were non-reactive in the microplate assay. These findings are similar to those observed with the tube LAI assay (101).

1.3.5 *Tube LAI assay*

The tube LAI assay was first described by Holan and coworkers in 1974 using various antigen extracts in the rat (71). They used soluble alloantigens and tumor extracts that were prepared by the extraction of normal tissues (spleen and muscle) or tumors (sarcomas RSL and LW 13K2) and peritoneal cells as LAI indicator cells. They detected specific alloimmune reactivity after skin grafting. This specific reactivity was maintained even across a xenogeneic barrier. Tumor specificity was

also observed, but the LAI seemed to manifest itself more strongly in rats with stationary tumors than in those with progressively growing tumors. This seems to be characteristic for this assay when used in tumor systems. Holan et al. (114) also observed a poor correlation between the LAI-reactivity and leukocyte activity as measured in the long term cytotoxicity assays (1.2.2.2.b).

The further development of the tube LAI assay since then, has been the work of Thomson and coworkers. The tube LAI assay as described by Grosser and Thomson (115) is performed in triplicate in 16 × 150 mm glass tubes. Each tube contains 1×10^6 viable leukocytes in 0.1 ml of medium, 0.1 ml of buffer or of specific tumor extract or unrelated tissue extract and 0.3 ml of medium. The tubes are then incubated horizontally so that the contents cover 75% of the lower surface of each tube, in a himidified CO_2 incubator at 37°C for 2 hours after which the tubes are placed vertically and their contents gently agitated. The nonadherent cells are sampled easily and then counted by manual, automated (115,116), enzymatic (112) or isotopic (79,80) means.

Putative mechanisms

In their initial study (71), Holan and coworkers indicated that the tube LAI system differed from the other LAI systems in that no lymphokine could be demonstrated. They suggested that the LAI was mediated by sensitized macrophage-like cells which possessed antigen receptor that may or may not have originated from some sort of cytophilic antibody.

The detailed analysis of the possible mechanism(s) of the tube LAI system has been performed by Thomson and his collegues. They verified many of the previous observations of Holan et al. (71) and extended them further. Thomson and Grosser (72) found that only mononuclear cells from peripheral blood leukocytes were reactive in the LAI. Systematic removal of either phagocytic cells, or cells with Fc receptors or monocytes from a reactive mononuclear cell suspension resulted in the abrogation of LAI-reactivity. T-cell enriched, monocyte-depleted preparations were equally nonreactive, whereas lymphocyte-poor, monocyte-enriched populations reacted well in this assay. Hence, it was concluded that the blood monocyte was the reactive cell in the tube LAI assay, but the source or specificity was not known. The findings of Grosser et al. (118) and Eccles et al. (119) supported the earlier finding of Holan et al. (71) that macrophage-like cells were involved in the tube LAI assay. Since no lymphokine seemed to be involved in the tube LAI assay (71,72,115), Marti et al. (120), investigated the role of monocytes and confirmed that no lymphokine was involved, but conclusively demonstrated that normal monocytes could be made specifically reactive to tumor extracts by means of a cytophilic IgG obtained from sera of the patients with the relevant tumor. In addition, they also demonstrated that arming of monocytes was optimal when sera from patients with moderate tumor burdens rather than those with disseminated disease were used. This observation is reminiscent of a similar observation in rats by Holan et al. (71). However, in their study, Holan et al. (71) observed that the macrophage reacted directly with antigen and not through a cytophilic antibody.

The absence of reactivity of cells from donors with tumor overload apparently relates to their inability to bind free cytophilic anti-tumor antibody (121). In addition, sera of such patients have no free cytophilic antibody. This is as a result of large quantities of tumor antigen in circulation and the formation of immune complexes (101). If the non-reactive cells of individuals with tumor overload are treated with trypsin (101,123) or with prostaglandin E2 (122), the ability to bind IgG and reactivity in the tube LAI assay is restored. This is because both trypsin and prostaglandin E2 increase the level of intracellular cyclic adenosine monophosphate (AMP) by activating the cell surface enzyme adenylate cyclase. The importance of circulating free antigen in human cancer may be crucial as an "escape" mechanism whereby the tumor evades the normal consequences of an immunological response.

An alternative putative mechanism of the tube LAI assay demonstrating the presence of an active lymphokine and the obligatory role of weakly adherent or nonadherent cells in humans and guinea pigs was reported by Yagawa and collegues (117). Although their LAI assay appeared to be similar to tube assay, it resembled the closest to microplate or hemocytometer assay. However, Tong et al. (123), using a tube LAI assay similar to that of Holan et al. (71), and Grosser and Thomson (115), could not confirm that an active lymphokine was involved. On the other hand, they found various cell types (T-cells, B-cells and other non-rosetted cells) reactive in the tube LAI assay. Their findings could not be satisfactorily evaluated since their methodology did not describe accurately the assay conditions that were used. In 1982, Thomson et al. (124,125) demonstrated the involvement of leukotrienes in the tube LAI assay, whereby the assay can be divided into three parts: immunologic recognition of tumor antigen, generation of leukotrienes from monocytes and leukotriene-induced inhibition of the adherence of leukocytes to glass (118,120,124,125). Adherence to substrate by leukocytes is generally regarded as an active cellular event (126), thus, tumor extract-induced inhibition of adherence to glass was viewed as a negation of an active cellular process. Thomson et al. (127), however, showed that the adherence of leukocytes to glass was a comparatively passive event, since neither oxidative metabolism, an intact cytoskeletal microtubular system, nor calcium movement was needed, whereas, tumor extract-induced LAI required all these cellular components. Since tumor extract-induced LAI depends upon the generation of leukotriene chemoattractants and since other chemoattractants added to normal leukocytes inhibit their adherence to glass, Thomson et al. (127) have proposed that the induced locomotion from glass would be more appropriately named as "mobility" instead of "adherence inhibition".

A micro-glass-tube LAI assay was used by Morizane and Sjögren (27,80) who demonstrated that both T-lymphocytes and monocytes functioned as indicator cells in the tube LAI using a rat colon adenocarcinoma model.

Recently, in 1984, Shenouda et al. (128) demonstrated that human T-cells react specifically to autologous cancer extract in the tube LAI assay and suggested that the tumor extract recognition could be major histocompatibility complex (MHC)

restricted. They confirmed this in studies (129) using monoclonal antibodies to class-I MHC antigens and observed that while the response of T-cells to the autologous cancer extract in the tube LAI was inhibited by the monoclonal antibodies, the antibody dependent response to allogeneic cancer extracts using the same assay in the same patient remained unaffected and therefore was not MHC restricted.

1.3.6 *Humoral LAI (H-LAI) assay*

An essentially different LAI assay was reported in 1981 by Kotlar and Sanner (130,131). Their "Humoral Leukocyte Adherence Inhibition test" (H-LAI) was performed using trypsinized peripheral blood leukocytes (PBL) from control subjects as indicator cells and 0.25% serum from the patient was added to the assay system together with the relevant tumor extract. Anti-tumor antibody in the patient's serum (if any) reacted specifically with the appropriate tumor antigen and by the reaction with Fc receptors of the Fc receptor bearing PBL caused their non-adherence. This assay was claimed to have accuracy, specificity and sensitivity comparable to the conventional LAI assay (131).

1.3.7 *Comparison of LAI assays with other assays*

Cell-mediated tumor immunity can be assessed using a variety of assays that have been developed over the years.

The delayed cutaneous hypersensitivity test (DCH) is the only in-vivo test, all others such as lymphotoxin (LTOX), various long term and short term microcytotoxicity assays (MCA), macrophage and leukocyte migration inhibition assays (MMI and LMI) and leukocyte stimulation assays are all in vitro tests. Practical disadvantages of some of these tests include the large number of reactive cells that are necessary, long assay incubation periods, tedious time consuming manipulations, subjective endpoint determinations and the requirement for tissue culture techniques necessary for cultured tumor cells. In comparison, the LAI assay is a relatively simple and fast assay.

Holt et al. (108) obtained better results with the microplate LAI test than with the LMI assay in detecting the primary and secondary responses to defined antigens in mice. In the same study, the development of spleen cell reactivity to TAA from B16 tumor, following subcutaneous inoculation with viable B16 melanoma cells in mice, was equally well detected by the MCA as by the LTOX and LAI assays. The leukocyte stimulation and LMI assays proved to be considerably less accurate. Overall, the LAI assay compared favourably with the other established techniques.

Rudczynski et al. (132) compared the microplate LAI and the LMI tests in breast cancer patients using breast carcinoma (cell line MCF-7) extract as antigen. The LAI assay had a positive response in 69% of the patients as compared to less than 50% using the LMI assay. There was no correlation between the LAI and LMI

indices or between the positive and negative responses. The results suggested that the two tests measured the production of different mediators (86,104) or that these mediators exert their effect on various cell populations (133,134). Fukada et al. (135) and McCoy et al. (136) reported high sensitivity of 75 to 84% using the LMI assay in breast cancer patients. They observed false positive reactions in 5 to 10% of the controls. Comparable LAI results were reported by Sanner et al. (137), Flores et al. (138) and Halliday et al. (139) in breast cancer with sensitivity ranging from 69 to 89%.

In colorectal cancer patients, Burtin et al. (140) obtained very poor results using the LMI assay. They observed a sensitivity of only 43% whereas false positive reactions were observed in 65% of the patients with non-malignant colorectal disorders. In sharp contrast to these results, Halliday et al. (141) and Tataryn et al. (142) reported excellent sensitivity of 95% and a specificity of 90% in colorectal cancer patients using the LAI assays.

Skin tests with tumor extracts may be useful in the diagnosis of malignant diaseases. The theoretical possibility, that the extracts contain viruses or other oncogenic components limit the use of these tests. Burger et al. (116) and Vetto et al. (143) compared the LAI assays with the DCH assay in a group of patients with melanomary squamous cell carcinoma of head and neck or neuroblastoma. They observed a good correlation between the LAI results and the dermal response to melanoma, epidermoid carcinoma and neuroblastoma tumor extracts. The sensitivity of the LAI tests appeared to be five to ten fold greater than that of DCH tests in terms of the amount of protein required to demonstrate a positive response. There was no correlation between the size of the dermal reaction and the LAI indices.

1.3.8 *Comparison of hemocytometer, microplate and tube LAI assays*

The originally described hemocytometer assay (66) is based on the adherence of cells to the surface of a hemocytometer. The only significant improvement in this assay has been the automation of the counting procedures by the use of electronic devices (77). This allows the collection of large amount of results which are necessary for the quantitative assessment of LAI data. The assay can be modified by using a specific lymphokine which has been generated previously by the interaction of leukocytes with tumor extract and had been stored. Another advantage of this assay is that it can be used to detect serum factors that block specific LAI-reactions (71,91,95-97).

In the studies of melanoma, colorectal carcinoma and breast cancer, this assay showed excellent specificity (139). However, a high proportion of patients with benign breast diasease reacted with the breast cancer extracts as well, though normal subjects or patients with unrelated tumors were unreactive. This suggests the existence of common antigens generating cell-mediated immunoreactivity in benign and malignant breast tumor patients. The hemocytometer assay differs from the microplate and tube LAI assays with regard to its sensitivity. Maluish et al.

(103) using the hemocytometer assay observed specific leukocyte activity at all stages of tumor growth. Noonen et al. (91) found the assay to be of no value in monitoring the patients during chemo- and/or immunotherapy since the results of the assay did not correlate with the clinical effect of these types of therapy.

The microplate LAI assay like the hemocytometer assay is sensitive to lymphokine(s) (104, 107), is inhibited by sera containing blocking factors (75) and can detect arming factors in the sera (105). Since, the LAI response in the microplate assay like the tube LAI assay can be abrogated by excessive circulating tumor antigens, there is a correlation of LAI response with the stage of the disease (74,132,144).

The microplate assay has been less well investigated than either the hemocytometer or the tube assays. There is also considerable disagreement in the reported results with different variations of the microplate LAI test. The putative mechanism(s) of this assay is also not yet satisfactorily resolved. Holt et al. (104) showed that in mice the effect is mediated by lymphokine produced by T cells, whereas Russo et al. (73) showed that in humans it is dependent on B cells and monocytes armed with antibody. Using the microplate assay, Fritze et al. (147) failed to discriminate between high-risk patients with and without breast cancer.

The tube LAI assay differs considerably from both the hemocytometer and the microplate LAI. The test is performed in tubes and in medium without serum. The adherence inhibition in this assay is lymphokine independent but does depend on the release of immunopharmacologic mediators similar to leukotrienes by leukocytes when they specifically react with tumor extract.

Although, the tube LAI has not been extensively demonstrated with defined antigens, several authors have obtained excellent results using crude allogeneic extracts (138,142,145,146). In addition, in these studies, the LAI response correlated well with the stage of the disease. The normally unreactive cells from patients with disseminated disease were made reactive in the tube LAI assay by pretreatment of their cells with prostaglandin E2 (122). The negative results with the tube LAI assay have also been reported by various authors (148,149). This assay was found to be unsuitable for the diagnosis of Huntington's chorea (148). Vose et al.(149) reported the failure of this assay to discriminate between benign and malignant breast disease. Although, the tube LAI assay on its own has a sensitivity of about 70%, it can be used very effectively in combination with carcinoembryonic antigen (CEA) determinations as reported recently by Payne et al. (150) who diagnosed colorectal carcinoma with 91% sensitivity and 68% specificity. Their results provide additional evidence that CEA antigens are not the antigens that trigger the LAI reaction.

1.4 The objectives of this study

For the past two decades, intensive search has been made for the existence of tumor-specific antigens of human cancer. The recent succesful development of

monoclonal antibodies against TAA on human cell membrane has not yet resulted in the identification of any tumor-specific determinant(s) on cancer cells.

An alternative approach for the identification of tumor-specific antigens has been to study the immune response of the host to cancer. Cell-mediated cytotoxicity was initially investigated using microcytotoxicity assays (47). Specific cytotoxicity against a variety of cultured cells from human tumors was observed with lymphoid cells from tumor-bearers or individuals whose tumor had been resected. The whole concept of specific cell-mediated cytotoxicity in human cancer was doubted when natural cytotoxicity was discovered (18,19). However, investigators working with the tumor extract-induced leukocyte adherence inhibition (LAI) phenomenon have successfully provided much of the existing evidence for specific anti-tumor immunity in animals and human cancers (81,82).

The ultimate objective of the present study was to use the tube LAI assay to monitor the purification of human TAA from crude tumor extracts. On the assumption that TAA are foreign or modified human major histocompatibility complex antigens (HLA antigens) or are closely associated with these antigens, the biochemical techniques used to study the nature of HLA antigens could be applied to obtain an insight into the biochemical nature of TAA and their relation (if any) to HLA antigens.

The tube LAI assay was chosen since it was claimed to be simple, rapid and reproducible. In the first instance it was necessary to develop a reliable tube LAI technique of high sensitivity. In the initial studies it was investigated whether this could be achieved by using partially purified tumor extracts. Since the amount of patient tumor material severely restricted the amount of crude extracts that could be chromatographed and purified further using other physico-chemical techniques, LAI studies in rats were also pursued.

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CHAPTER 2

EVALUATION OF THE TUBE LEUKOCYTE ADHERENCE INHIBITION (LAI) ASSAY IN PATIENTS WITH BREAST CANCER AND GASTROINTESTINAL CANCER*

2.1 Introduction

The leukocyte adherence inhibition (LAI) assay is a test for cell mediated immunity and has been used to investigate immune reactivity to a broad spectrum of antigens in man and experimental animals (1-5). LAI has been used in studies of immune recognition in cancer patients and has shown a high degree of sensitivity and reproducibility. The LAI assay is based on the observation that leukocytes from cancer patients lose their ability to adhere to glass or plastic surfaces when incubated in vitro with extracts of tumors from the same organ and histogenesis. In the tube LAI assay, the cell which had lost the adherence capacity was demonstrated to be a monocyte containing cytophilic anti-tumor antibody (6). It has also been reported that serum of patients with large tumor burdens contains free tumor antigen that coats the reactive leukocytes, thus abolishing their LAI reactivity (7). Previous studies (8) have led to the consensus that LAI assay could be a valuable aid for the monitoring of cancer patients since it has the required properties of specificity and correlation with the stage of the disease.

In the current pilot studies, the tube LAI assay was used to investigate human tumor immunity in blood samples from 41 cancer patients and 17 controls.

2.2 Materials and methods

2.2.1 *Patients*

All patients were from Dijkzigt hospital, Rotterdam. They consisted of 15 patients with adenocarcinoma of the breast, 5 with benign breast tumors, 11 with colorectal cancer, 8 with stomach cancer and 2 with pancreatic cancer. The control group consisted of 17 patients suffering from various non-malignant diseases.

The diagnosis as confirmed by histological examination was known before LAI assays were performed. The clinical stage the patients were in was not taken into account in evaluating the LAI results.

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2.2.2 *Preparation of tumor extracts*

Extracts of cancers of the breast, colon, stomach and pancreas were prepared from samples after surgical excision. Fatty and fibrous tissue were dissected away and the specimen was cut into small pieces in RPMI-1640 medium (GIBCO). The material was homogenized for 5 minutes in 4 volumes of RPMI medium using an ultra-turrax TR18-10 homogenizer. The homogenate was centrifuged at 1000G for 5 minutes and subsequently at 20000G for another 60 minutes. The supernatants (tumor antigen extracts) were stored at -70°C in 2 ml aliquots and were used only once. The protein concentration of the stock extracts was determined by the method of Lowry (9) and ranged from 2 to 4 mg/ml.

2.2.3 *Isolation of peripheral blood leukocytes (PBL)*

Samples of blood from each patient were collected in heparinized 10 ml tubes. Peripheral blood lymphocytes were isolated by gradient centrifugation (10). Briefly, blood was diluted 1:1 with RPMI 1640 medium (GIBCO), layered over lymphocyte separation medium (LSM; Litton Bionetics) and centrifuged for 15 minutes at 500G. Cells at the plasma/LSM interface were aspirated and washed twice in RPMI medium. After counting and viability testing using trypan blue dye exclusion, the suspension was adjusted to a concentration of 5×10^6 living cells per ml.

2.2.4 *Tube LAI assay*

The LAI assay was performed in triplicate in 12.5 ml glass round bottom tubes as described previously (2). Each tube contained 0.3 ml RPMI medium, 0.1 ml of the leukocyte suspension (5×10^5 cells), and 0.1 ml (100 μg protein) of the tumor extract. The tubes were placed in a near horizontal position, so that the contents covered about 80% of the lower surface. They were incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air. After two hours the tubes were placed in a vertical position and their contents mixed gently. A sample was withdrawn and the nonadherent cells were counted electronically in a microcell counter (TOA medical electronics, Japan). The results were expressed as the nonadherent index (NAI) which was calculated using the formula:

$$NAI = \frac{A - B}{B} \times 100$$

where A represents the number of nonadherent cells in the presence of specific antigen and B stands for the number of nonadherent cells in the presence of non-specific antigen.

2.3 Results

2.3.1 *Non-specific inhibition of adherence*

When PBL from cancer patients or control subjects were incubated for 2 hours in the absence of antigen, about 15% of the cells were generally nonadherent. The addition of tumor extract caused non-specific inhibition of adherence which increased proportionally with protein concentration. Table 1 depicts the representative results of a dose-effect study performed with PBL of a patient with stomach cancer using colon tumor extract.

Table 1. Percentage of nonadherent leukocytes per tube LAI at different control tumor antigen concentrations.

Protein concentration (μg protein per tube)	% Nonadherence
300	58
150	40
75	34
37	22
18	14
—	12

2.3.2 *Antigen titration*

Table 2 shows the results of experiments in which the breast and colon cancer extracts were titrated against PBL from controls and patients with the corresponding type of tumor to determine the protein concentration at which there was an optimal discrimination between the specific and the non-specific inhibition of adherence.

Table 2. Specificity of breast and colon tumor extracts at various protein concentrations.

Protein concentration	Nonadherent Index (NAI)			
	breast-cancer		colon-cancer	
(μg protein per tube)	patient	control	patient	control
350	14	−12	15	0
175	75	24	48	−14
83	116	18	59	30
42	52	−16	35	17
21	−6	−16	14	−21

NAI values for breast cancer patients and controls were calculated using colon tumor extract as *non-specific antigen.* NAI values for colon patients and controls were calculated using breast cancer tumor extract as the *non-specific antigen.*

It was observed that, for both the breast and colon cancer extracts, the highest NAI values were found between 42 and 175 μg protein per tube. This is in agreement with earlier reports where a similar optimal protein concentration was observed for a variety of tumor extracts (11,12). Consequently all tube LAI assays were performed using a protein concentration of 100 μg/tube.

2.3.3 *NAI values for control subjects*

Seventeen control subjects were tested against tumor extracts as shown in Table 3. The colon tumor extract was used as the non-specific antigen in the group of controls which were tested with breast tumor extract; for other controls, breast tumor extract was used as non-specific antigen. The NAI values were found to range from −18 to +22. The mean NAI value of all controls tested was -1.8 ± 13.3. NAI values outside the limits of mean ± twice the standard deviation of the control population were considered to represent a significant effect. Therefore, a NAI $\geqslant 30$ was considered as positive and one of <30 as negative. The same cut-off point was chosen by others using the tube LAI assay (2,11,12).

Table 3. Nonadherent Index (NAI) values in 17 controls.

Number of controls	Test antigen	NAI	Mean NAI
6	Breast cancer	−22 + 15	− 4
5	Colon cancer	−6 + 22	+2
4	Stomach cancer	−31 + 19	+7
2	Pancreatic cancer	−18 + 6	−12

The tube LAI assay was performed using colon tumor extract as the *non-specific antigen* for the 6 breast cancer controls; the other groups were assayed using breast cancer extract as the *non-specific antigen*. The protein concentration used was 100 μg/tube.

2.3.4 *Selection of tumor extracts*

Initially, a total of 12 tumor extracts were prepared. These comprised, 3 breast tumor extracts, 4 colon tumor extracts, 3 stomach tumor extracts and 2 pancreatic tumor extracts. On prescreening these 12 tumor extracts, some preparation caused excessive nonadherence of control PBL or showed only weak antigenic effects when tested with PBL of patients that were reactive with extracts of the same type of tumor. Such extracts were discarded. Two breast tumor extracts, 1 colon tumor extract, 3 stomach tumor extracts and 1 pancreatic tumor extract were found to be useful.

It was also observed that extracts that were initially highly discriminating deteriorated with time. This has been previously observed by others (13). One breast tumor extract and two stomach tumor extracts had lost their activity after 4

months. This observation made it imperative to renew the tumor extracts regularly and test them together with older effective preparations.

2.3.5 *Clinical results of the tube LAI assay*

A summary of the results obtained using the tube LAI assay in 41 cancer patients and 17 controls are shown in Table 4. The results are expressed as the number of patients which showed NAI values of ≥30. The mean NAI values of the various groups are also dipicted. A NAI value of ≥30 was accepted as positive whether or not the difference in nonadherence of the two tumor extracts used was statistically significant.

Table 4. Summary of patients tested using the tube LAI assay for reactivity to various tumor extracts.

Tumor type	Number of patients tested	Positive in LAI*	Mean NAI
Breast cancer	15	9 (60%)	62
Benign breast disease	5	1 (20%)	21
Controls	6	0 (0%)	−4
Colon cancer	11	8 (72%)	39
Controls	5	0 (0%)	2
Stomach cancer	8	5 (63%)	47
Controls	4	1 (25%)	7
Pancreatic cancer	2	2 (100%)	56
Controls	2	0 (0%)	12

*A NAI value of ≥30 was considered as positive. NAI values for breast cancer patients were calculated using the extract of colon cancer as the *non-specific antigen*. NAI values for colon, stomach and pancreatic cancer patients were calculated using breast cancer extract as the *non-specific antigen*.

Positive LAI-reactions were observed in 60% of the breast cancer patients, in 72% of the colon cancer patients, and in 63% of the stomach cancer patients. The mean NAI (including the positive and negative NAI values) ranged from 39 to 72. The highest NAI values were found in the group of breast cancer patients, where NAI values of 200 were sometimes observed. The lowest values were observed in the group of colon cancer patients.

From the 5 patients with benign breast disease, only one patient reacted positively in the LAI with a relatively high NAI value of 77; the other 4 patients had NAI values of 20, 14, 0 and −6 respectively (mean 21).

False positive reactions were recorded in 2 of 15 control patients with non-neoplastic disease (13.3%). six out of the 15 control patients were females, 4 of whom were multiparous. None of these 4 multiparous women were LAI-positive.

This indicated that sensitization with alloantigens was unlikely to be involved in the LAI. However, the possibility that sensitizing agents such as blood transfusions and viral infections could play a role in the LAI cannot be totally excluded.

2.4 Discussion

The peripheral blood leukocytes (PBL) from breast and gastrointestinal cancer patients showed a reduced adherence to glass in the presence of allogeneic tumor extracts. This inhibition of adherence was tumor-type specific in the majority of the cases. The reduced adherence was not due to toxicity of the tumor extracts, since adherence of control PBL was not affected. In addition, immunization by alloantigens could be excluded as the cause of adherence inhibition, since the 4 multiparous controls that were tested, showed no reduced adherence. Sensitization in vitro to histocompatibility antigens present in the tumor extracts can also be excluded, since the interaction between PBL and antigens was brief.

PBL from control subjects showed non-specific nonadherence in the presence of tumor extract; this nonadherence was related to the protein concentration of the tumor extract. It was therefore essential to titrate the various tumor extracts and use a protein concentration which allowed a clear discrimination between specific and non-specific inhibition of adherence. By interpolation, a concentration of 100 μg per tube was chosen. This is similar to the protein concentration used by others (2,11,12).

In this study, about 13% of the controls were positive in LAI. This is higher than that reported by others (12,14). Generally, around 5% of the controls are found to react positively in the LAI. Since, the control group in the present study is somewhat small, no definite conclusion can be drawn with regard to the significance of the observed differences.

The PBL of 70% of both breast and gastrointestinal cancer patients reacted positively with the relevant allogeneic tumor extracts. This percentage of positive tumor-specific LAI-reactivity is lower than those reported by Tataryn et al. (13) and Halliday et al. (15). The results from these groups indicate that under optimal conditions, positive LAI results can be observed in 80-95% of the cases. However, the highest scores were found in patients with early stages of malignancy and the percentage of positive LAI-reactions decreased dramatically in disseminated stages, with a range of 30-60%, depending on the type of cancer under investigation and the degree of dissemination (11,13). Since, the number of patients in the experimental groups in the present study is rather small, it is premature to relate the LAI results with the stage of disease. Consequently, the current results represent pooled observations of various clinical stages of cancer. When this pooling-effect is taken into account, an overall positive LAI score of 70% is promising.

Currently, it was observed that one out of 5 patients with benign breast disease reacted positively in the tube LAI assay. Similar results have been reported in earlier studies in which positive LAI-reactions were observed in 10-24% of women with benign breast disease (7,11,14,16). It has been suggested that this relatively

high score in false positive reactions is due to antigenic similarities between dysplastic lesions of the breast and breast cancer tissue. The biological implications of these findings may be important, as it is known that patients with a histological picture of benign mammary dysplasia have a higher incidence of breast cancer (17,18,19).

From the results presented here, it can be concluded that the tube LAI assay is a valuable test for studying various aspects of tumor immunity. However, the tube LAI assay can only be executed successfully provided it is performed routinely and tumor extracts are carefully selected and regularly renewed.

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CHAPTER 3

TUBE LEUKOCYTE ADHERENCE INHIBITION (LAI) ASSAY IN PATIENTS WITH NON-MALIGNANT DISORDERS OF THE COLON AND COLORECTAL CANCER*

3.1. Introduction

In the preceeding chapter, the tube LAI assay was used successfully to demonstrate tumor-specific LAI-reactivity in breast and gastrointestinal cancer patients. However, the assay was found to have two main limitations. These were a) an extensive prescreening of the extracts was imperative to select extracts that discriminated well in the assay and b) crude extracts that were initially highly discriminating deteriorated on prolonged storage at -70°C. Thus, old extracts had to be regularly replaced with new ones.

The studies described in this chapter were performed in an attempt to try to overcome these two main drawbacks. The aim was to determine whether the sensitivity of the assay could be improved by extracts that contained less sub-cellular impurities than those used in the studies in chapter 2 and whether such extracts showed an improved "shelf-life". Patients with colitis, various types of polyps of the large bowel and colorectal cancer were tested. The ultimate goal was to use the tube LAI assay to follow the purification of human tumor-associated antigens (TAA) from crude tumor extracts.

3.2. *Materials and Methods*

3.2.1. *Patients*

All individuals were patients at Dijkzigt hospital, Rotterdam. Twenty nine patients with colorectal cancer, 12 patients with colitis and 21 patients with various types of polyps of the large bowel were evaluated. The control groups comprised 18 healthy volunteers and 29 patients with various non-gastrointestinal diseases.

All diagnosis were known prior to testing in the LAI assay and those of colorectal cancer were confirmed by histological examination. The clinical stage of the disease was not taken into account for the final evaluation of the results.

3.2.2. *Preparation of tissue extracts*

Colorectal and breast cancer tissues were obtained at surgery. Tissue extracts were prepared using the basic method described in 2.2.2, with some modiciations. After fatty and fibrous tissue was removed, a 20% (w/v) homogenate of the tumor tissue

*LAI studies in colorectal cancer patients under recombinant leukocyte A interferon therapy were published in Oncology, 1985, 42, 157-163.

was prepared in ice-cold phosphate buffered saline (PBS) using an ultra-turrax TR 18-10 homogenizer. The homogenate was centrifuged at 1000G for 5 minutes and subsequently at 50000G for another 60 minutes. The supernatant (crude tumor antigen extract) was sterilized by filtration through 0.2μ millipore filter. The protein concentration of the stock extracts was determined using the Bio-rad protein assay (Bio-rad, Holland) and ranged from 3 to 7 mg/ml. All extracts were stored at –70°C in 1 ml aliquots and were used only once.

3.2.3. *Isolation of peripheral blood leukocytes (PBL)*

Twenty ml heparinized blood was obtained from each patient. PBL were isolated by gradient centrifugation as described in 2.2.3. After counting and viability testing using trypan blue, the cell suspension was adjusted to a concentration of 5×10^6 living cells per ml.

3.2.4. *Tube LAI assay*

The tube LAI assay was performed as described under 2.2.4. The results were expressed as nonadherent index (NAI). The NAI values outside the limits of mean $\pm$ twice the standard deviation of the control groups were considered to represent a significant effect.

3.3. Results

3.3.1. *Selection of tumor extracts*

Thirty colorectal cancer extracts and 19 breast cancer extracts were prepared. When the 49 extracts were prescreened, several extracts caused either excessive nonadherence of the PBL of healthy controls or showed only weak antigenic effects when incubated with PBL of patients that were reactive with other extracts of the same histological type of tumor. Such extracts were discarded. Twenty four colorectal and 16 breast cancer extracts were found useful.

In addition, it was observed that 20 of the colorectal and 14 of the breast cancer extracts had lost their LAI-reactivity within 4 months after preparation. The remaining 4 colorectal and 2 breast cancer extracts lost all reactivity 5 months after preparation. Similar observation have been reported earlier by others (1).

3.3.2. *Nonadherent Index (NAI) values in control groups*

Eighteen healthy volunteers and 29 patients with various non-gastrointestinal diseases were tested using colorectal cancer extract as specific antigen and breast cancer extract as non-specific antigen. The NAI values obtained in both the control groups are dipicted in Fig. 1. The NAI values in the 18 healthy volunteers ranged from +39 to –28 with a mean of –0.2 $\pm$ 17 (lane 4). The NAI values in 29 patients with various non-gastrointestinal diseases ranged from +35 to –23 with a mean of +2 $\pm$ 16 (lane 5). NAI values outside the limits of mean $\pm$ twice the standard deviation

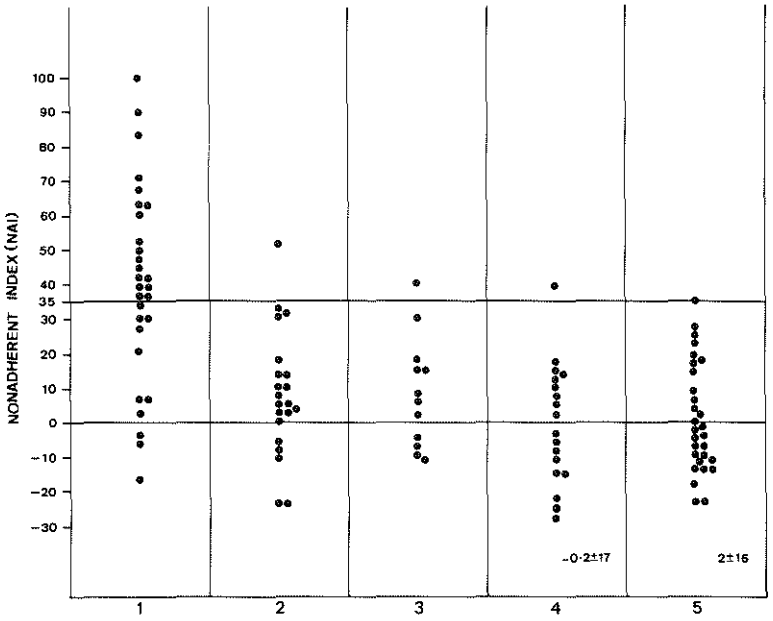

Figure 1. Distribution of NAI values in 29 colorectal cancer patients (1), 21 patients with polyps of the large bowel (2) and 12 patients with colitis (3).

The NAI values were calculated using colorectal cancer extracts as *specific antigen*, and breast cancer extract as *non-specific antigen*.

The NAI values outside the limits of mean ± twice the standard deviation of the control groups consisting of healthy individuals (4) and patients with various non-gastrointestinal diseases (5) were considered to have a significant effect. Consequently, NAI values of ⩾35 were regarded as positive.

of the control groups were considered to represent a significant effect. Consequently, a NAI value of ⩾35 was considered as positive and one of <35 as negative. This cut off point is slightly higher than those reported by others (2-4).

3.3.3. *Clinical results*

The results in the 3 groups of patients with non-malignant disorders of the colon and colorectal cancer are also shown in Fig. 1. The tube LAI assay was performed using colorectal cancer extract as specific antigen and breast cancer extract as non-specific antigen. A NAI value of ⩾35 was regarded as positive.

Positive LAI-reactions were observed in 62% (18 of 29) of the colorectal cancer patients (lane 1), in 5% (1 of 21) of the patients with various types of polyps of the large bowel (lane 2) and in 8% (1 of 12) of the patients suffering from colitis (lane 3). A further subclassification of the patients with various types of polyps of the large bowel and patients suffering from colitis are shown in Table 1. The mean NAI value of the 3 groups (including positive and negative NAI values) ranged from +8 to

+40. The highest NAI values were observed in the colorectal cancer patients. It is seen in Table 2 that there was no correlation between the LAI results and the degree of differentiation of the tumor in the colorectal cancer patients.

False positive reactions were observed in 1 of 18 (6%) healthy volunteers and in 1 patient of 29 (3%) with non-gastrointestinal diseases. False negative reactions were observed in 11 of 29 (38%) colorectal cancer patients.

Table 1. Summary of LAI results in patients with non-malignant disorders of the colon.

Diagnosis of leukocyte donor	Number tested	Number LAI positive
1. Polyps of the large bowel:		
a) Hyperplastic	2	0
b) Adenoma	4	0
c) Multiple polyps	10	0
d) Familiar polyposis coli	1	0
e) others	4	1*
2. Colitis:		
a) Crohnse colitis	4	1
b) Colitis ulcerosa	2	0
c) Undefined colitis	6	0

*This patient had undergone polypectomy, but no lesions were observed by colonoscopy.

Table 2. A comparison of LAI results and degree of tumor differentiation in colorectal cancer patients.

Degree of colon tumor differentiation	Number of patients	
	LAI positive (NAI $\geq$35)	LAI negative (NAI $<$35)
Poorly differentiated tumors	5	1
Moderately differentiated tumors	5	7
Well differentiated tumors	3	1

A total of 22 colorectal cancer patients were evaluable. There is no significant difference between any of the three differentiation catagories.

3.4. Discussion

The main objectives that were persued in the investigations presented here were to increase the sensitivity of the tube LAI assay by using tumor extracts containing minimal sub-cellular impurities and to assess whether such extracts had an extended "shelf-life" than those used previously (5). A total of 49 extracts were prepared and were prescreened with PBL of healthy volunteers. Nine extracts produced excessive nonadherence and were discarded. The majority of the remaining usable extracts had lost their reactivity within 4 months of preparation. This period was similar to that observed previously (5) and would plea for a better

isolation technique in which the amounts of the lysozomal enzymes which could be responsible for the limited "shelf-life" of the extracts were kept at a minimum. One such approach would be the enzymatic stripping of the antigenic determinants from the tumor cell surface and/or further purification of the extracts which may yield material of an extended "shelf-life".

With regards to the specificity of the LAI assay using these crude extracts, it was observed that 6% (1 of 18) of the healthy volunteers and 3% (1 of 29) of the patients with non-gastrointestinal diseases showed positive reactions with allogeneic colorectal cancer extracts. These values are in agreement with those observed by others (4, 6). In those studies, an average of 5% of the controls were found to react positively in the tube LAI assay.

Sixty two percent (18 of 29) of the colorectal cancer patients reacted positively in the LAI assay. This percentage is considerably lower than that reported by others (1, 7). In those reports, between 80 and 95% of the patients that were tested reacted positively. The positive score rate in the LAI has been shown to be related to the clinical stage of the disease (1, 3, 8). Highest percentage of positive LAI-reactions were observed in patients with an early stage malignancy. This percentage dropped considerably and ranged from 30-60% in patients with late stage malignancies according to the type of cancer. The results reported here are in agreement with our previous results where 70% of the patients with colorectal cancer showed positive LAI-reactions (9). The false negative results in 38% (11 of 29) of the present colorectal cancer patients could be due to either the inherent insensitivity of the tube LAI assay, or due to a large tumor burden in these patients whereby the excess circulating tumor antigen in the serum coats the reactive leukocytes rendering them unresponsive in the LAI (10).

In the two groups of patients with non-malignant disorders of the colon, 5% (1 of 21) of the patients with various kinds of polyps of the large bowel and 8% (1 of 12) of the patients suffering from colitis reacted positively with allogeneic colorectal cancer extracts. Since, these percentages are comparable to those observed in the two control groups, they can be considered as false positives.

In conclusion, although the sensitivity of the tube LAI assay could not be improved by the use of extracts with minimal sub-cellular impurities, the high number of false positive reactions observed in the controls of the previous study (5) were reduced from 13% to an acceptable level of 5% by the use of such extracts. In other words, although the sensitivity of the assay was not improved, there was a tendency that its specificity was. It would therefore be worthwhile to investigate whether further purification of the crude extracts by column chromatography would ultimately result in an increase in the sensitivity while retaining the high specificity of the assay.

3.5.**References.**

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CHAPTER 4

TUBE LEUKOCYTE ADHERENCE INHIBITION (LAI) ASSAY IN COLORECTAL CANCER PATIENTS USING PARTIALLY PURIFIED TUMOR EXTRACTS

4.1. Introduction

The tube leukocyte adherence inhibition (LAI) assay was shown to have a sensitivity of 62% in colorectal cancer patients in the preceeding chapter. This percentage was considerably lower than that reported previously by others (1, 2) and therefore limits its clinical value for the routine screening of cancer patients. However, at the same time, the studies described in the previous chapter showed that the specificity of the assay was increased to 95% as compared to 87% in the earlier studies (3). This seems to indicate some reduction in the number of false positive reactions.

In the studies described in this chapter, an attempt was made to increase the sensitivity of the assay while retaining its high specificity by using partially purified extracts. Such extracts were obtained by sephacryl S-200 column chromatography of the crude tumor extracts. It was also examined whether such partially purified extracts showed an extended "shelf-life" as compared to their crude counterparts since considerable amounts of cellular lysozomal enzymes which could be responsible for the limited "shelf-life" would have been eliminated.

The tube LAI assay was performed in colorectal cancer patients using partially purified extracts of colorectal and breast cancers.

4.2. Materials and Methods

4.2.1. *Patients*

Eleven patients with colorectal cancer were evaluated. The control group consisted of 12 healthy volunteers and 10 patients with non-gastrointestinal diseases.

The diagnosis of colorectal cancer was known prior to testing in the LAI assay and was confirmed by histological examination. The clinical stage of the disease was not taken into account for the final evaluation of the results.

4.2.2. *Preparation of tissue extracts*

Colorectal and breast cancer extracts were prepared as described in 3.2.2.

4.2.3. *Sephacryl S-200 column chromatography*

After the 50000G fraction had been concentrated, a 1 ml sample containing approximately 30 mg of protein was applied to a calibrated K26/70 sephacryl S-200 column (Pharmacia) and eluted with PBS (pH 7.3) at a flow rate of 10 ml/hr. The eluate was concentrated approximately five fold using the "on-line amicon thin channel system". Four fractions were collected from the column: A high molecular fraction designated "F 120", moving behind the excluded volume of the column; a second fraction eluting slightly ahead of where a haemoglobin/heptoglobin standard eluted, designated "F 100", a protein peak eluting ahead of the area where a bovine serum albumin (BSA) standard eluted, designated "F 68" and a low molecular weight fraction eluting in the area where an ovalbumin standard eluted, designated "F 48". All the four fractions were tested in the LAI.

4.2.4. *Isolation of peripheral blood leukocytes (PBL)*
PBL were isolated from 20 ml heparinized blood from each patient as described in 2.2.3. After the cell suspension was counted and its viability tested using trypan blue, its concentration was adjusted to 5×10^6 living cells per ml.

4.2.5. *Tube LAI assay*

Tube LAI assay was performed using the same conditions as described in 2.2.4. The LAI results were expressed as nonadherent index (NAI). The NAI values outside the limits of mean $\pm$ twice the standard deviation were considered to represent a significant effect.

4.3. Results

4.3.1. *Selection of fraction reactive in the LAI*

When crude extracts of breast or colorectal cancer were subjected to sephacryl S-200 column chromatography, four peak fractions "F 120", "F 100", "F 68" and "F 48" were obtained. The elution pattern is shown in Fig. 1. When these four fractions

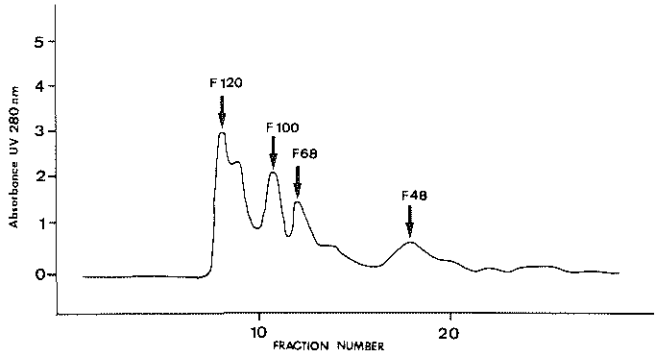

Figure 1. Molecular sieve chromatography of crude extracts of colorectal and breast cancers. The concentrated crude material was applied to a calibrated K26/70 Sephacryl S-200 column and eluted with PBS pH 7.3. "F 120" had the most LAI-reactivity.

were tested for LAI-reactivity, it was observed that most LAI-reactivity was present in the "F 120" fraction. These results are summarized in Table 1. Similar observations were reported previously by Thomson et al. (4) who demonstrated that most LAI-reactivity was present in the high molecular weight fraction which was obtained by sephadex G-150 column chromatography of solubilized extracts of human tumors.

Table 1. NAI values in patients using "F 120", "F 100", "F 68" and "F 48" peak fractions

Patient no	Diagnosis	NAI-values "F 120"	"F 100"	"F 68"	"F 48"
1	colorectal cancer	+42	0	−12	−40
2	colorectal cancer	+41	+26	0	0
3	non-gastrointestinal disease	+19	+10	−2	−6
4	non-gastrointestinal disease	+28	+16	−9	−14

NAI values were calculated using peak fractions "F 120", "F 100", "F 68" and "F 48" from colorectal cancer extract of proven specificity as *specific* antigens and the respective breast cancer fractions as *non-specific* antigens.

"F 120" was therefore designated as partially purified extract and was used in all experiments. A total of 18 colorectal and 6 breast cancer extracts were partially purified and tested. Since the total quantity of each extract was limited owing to their low protein concentration, only 2 colorectal cancer extracts could be tested beyond a 3 month period. Both these extracts had lost their LAI-reactivity when tested 4 months after preparation. This suggested that the partially purified extracts had no improved "shelf-life" as compared to their crude counterparts.

Preliminary results of immunoprecipitation of partially purified extracts using rabbit-anti-human β2-microglobulin antiserum and the analysis of the precipitates using sodium dodecyl sulphate-polyacrylamide gel dectrophoresis (SDS-PAGE) indicated the presence of β2-microglobulin in these extracts. This is in agreement with the observations previously made by Thomson et al. (4).

4.3.2. *LAI results using partially purified extracts*

The NAI values obtained in 11 colorectal cancer patients and 2 control groups are shown in Fig. 2. The two control groups comprised 12 healthy volunteers and 10 patients with non-gastrointestinal diseases. The tube LAI assay was performed using partially purified colorectal cancer extract as specific antigen and partially purified breast cancer extract as non-specific antigen.

The NAI values in the 12 healthy volunteers ranged from −5 to +20 with a mean of +10 ± 7 (lane 2). The NAI values in the 10 patients with various non-gastrointestinal diseases ranged from −24 to +15 with a mean of −4 ± 14 (lane 3). NAI values exceeding the limits of mean ± twice the standard deviation of the

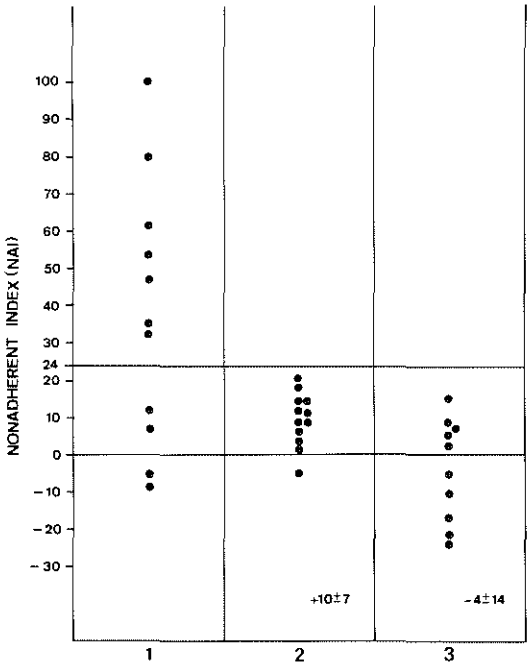

Figure 2. Distribution of NAI values in 11 colorectal cancer patients (1).
The NAI values were calculated using partially purified colorectal cancer extract as *specific antigen*, and partially purified breast cancer extract as *non-specific antigen*.
The NAI values outside the limits of mean ± twice the standard deviation of the control groups (2) and (3) were considered to have a significant effect. Consequently, NAI values of ≥24 were regarded as positive.

control groups were considered to have a significant effect. Consequently, a NAI value of ≥24 was regarded as positive and one of <24 as negative.

Seven out of the eleven (64%) colorectal cancer patients showed positive LAI-reactivity (lane 1). The NAI values ranged from −5 to +100 (mean +38±35).

In neither of the two control groups false positive reactions were observed. False negative reactions were observed in 4 of 11 (36%) colorectal cancer patients.

4.3.3. *Comparison of LAI results obtained using crude and partially purified extracts*

Fig. 3 shows the LAI results obtained in a separate group of 6 colorectal cancer paients and the two control groups which comprised 10 healthy volunteers and 19 patients suffering from non-gastrointestinal diseases. Tube LAI assay was performed using simultaneously, crude colorectal cancer extract and its partially purified counterpart as specific antigens and the crude breast cancer extract and its partially purified counterpart as non-specific antigens respectively.

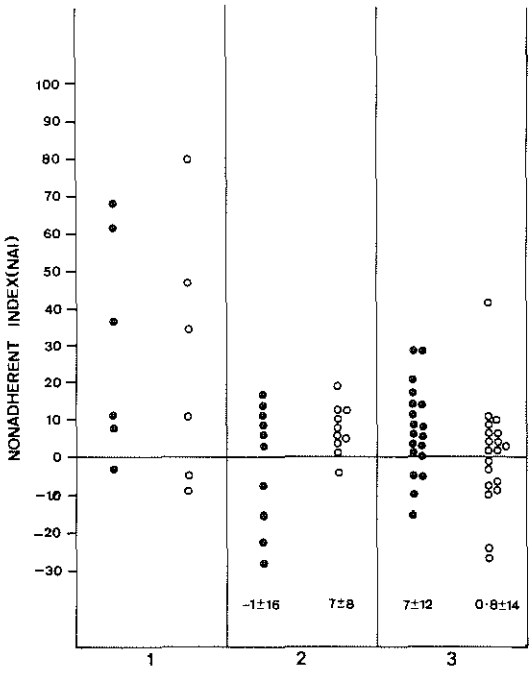

Figure 3. Distribution of NAI values in 6 colorectal cancer patients (1).

The NAI values were calculated using crude (●) colorectal cancer extract and its partially purified (o) counterpart as *specific antigens* and crude breast cancer extract and its partially purified counterpart respectively as *non-specific antigens.*

The NAI values outside the limits of mean ± twice the standard deviation of the control groups (2) and (3) were considered to have a significant effect. Consequently, NAI values of ⩾31 using the crude extracts and ⩾26 using the partially purified extracts were regarded as positive.

The NAI values in the 10 healthy volunteers (lane 2) ranged from -28 to +17 (mean -1 ± 16) using the crude colorectal cancer extract and from -6 to +20 (mean +7 ± 8) using its partially purified counterpart. The NAI values in the 19 patients with non-gastrointestinal diseases (lane 3) ranged from -15 to +29 (mean +7 ± 12) using crude colorectal cancer extract and -27 to +14 (mean +0.8 ± 14) using its partially purified counterpart. NAI values exceeding the limits of mean ± twice the standard deviation of the control groups were considered to have a significant effect. Thus, a NAI value of ⩾31 using crude colorectal cancer extract and ⩾26 using its partially purified counterpart were regarded as positive.

Three out of the 6 (50%) colorectal cancer patients reacted positively with allogeneic crude colorectal cancer extract and its partially purified counterpart. The NAI values ranged from -3 to +68 (mean +30 ± 30) and -9 to +80 (mean +27 ± 34) respectively.

No false positive reactions were observed in either of the control groups using crude colorectal cancer extracts, whereas 1 out of 19 (5%) patients suffering from non-gastrointestinal diseases reacted positively with its partially purified counterpart. False negative reactions were observed in 3 of 6 (50%) colorectal cancer patients both using the crude colorectal cancer extract and its partially purified counterpart.

4.4. **Discussion**

The present investigations were undertaken in order to examine whether the sensitivity of the tube LAI assay could be increased by using partially purified extracts and to assess whether these extracts had a "shelf-life" exceeding 4 months.

A total of 24 tumor extracts were partially purified using sephacryl S-200 column chromatography and tested. Since most of these extracts had low protein concentration, most of them were used up within 3 months after preparation. However, 2 partially purified extracts could be tested beyond this period, and both of them were found to have lost their LAI-reactivity within 4 months after preparation. This period was identical to that observed earlier for the crude extracts (3) and suggested that the partial purification of the crude extracts does not lead to an extended "shelf-life". One possible explanation for the limited "shelf-life" of the tumor extracts as opposed to the prolonged "shelf-life" of the intact tumor could be that once the intact tumor cells are disrupted, the LAI-reactive tumor-associated antigens on the tumor cell surface are displaced from their protective environment on the cell surface by loss of their quaternary structure. This loss results in them becoming susceptible to proteolytic enzymes leading to a gradual but permanent loss of LAI-reactivity within a finite period of time. Apparently, this rate of loss of reactivity cannot be slowed down by partial purification of the crude extracts on sephacryl S-200 columns.

None of the 12 healthy volunteers or 10 patients suffering from non-gastrointestinal diseases reacted positively with partially purified extracts of colorectal cancer (Fig. 2). One of 19 (5%) patients suffering from non-gastrointestinal diseases reacted positively with partially purified colorectal cancer extract (Fig. 3). However, the same patient showed no positive reaction with the crude extract. This high specificity of the tube LAI assay using partially purified extracts is similar to that observed previously by others (5, 6) where an average of 5% of the controls reacted positively with crude extracts.

Sixty four percent (7 out of 11) of the colorectal cancer patients reacted positively using the partially purified extract. This figure is slightly better than that observed using the crude colorectal cancer extracts where 62% of the colorectal cancer patients reacted positively indicating a slight but insignificant improvement in the sensitivity of the assay. This was confirmed in a separate series of experiments in which six colorectal cancer patients were simultaneously tested using crude colorectal cancer extract and its partially purified counterpart. In both instances 3

out of the 6 patients (50%) showed positive LAI-reactions confirming that the partial purification of the crude extract does not lead to a higher sensitivity of the assay. Similar results have been previously reported by others (7, 8) who observed that the use of purified antigen extracts did not result in a higher number of positive LAI-reactions.

The low protein concentrations of the partially purified extracts placed severe restrictions on the number of patients that could be tested since, considerable amounts of these extracts were used up for the determination of protein concentration for the optimum LAI-reactivity. Such limitations on the use of purified antigen extracts were previously reported by Thomson et al. (7). The amounts of tumor material available from patients undergoing surgery also imposed restriction on the amounts of crude extracts that could be chromatographed on Sephacryl S-200 columns and purified further using other physico-chemical methods.

Hence, in order to persue the initial objectives of increasing the sensitivity of the LAI assay and to improve the durability of the cancer extracts, it became essential to have a continuous source of large amounts of tumor material. Two possible approaches can be considered. The first would be a suitable animal tumor model which not only would act as a continuous source of tumor material, but could also be used to examine and improve conditions necessary for a more sensitive and reliable tube LAI assay. The second approach would be to use an appropriate human tumor cell line as a continuous source of LAI-reactive material which is either shed in the medium or obtained by solubilization of cell membrane antigens. The first approach is considered in chapters 5 and 6.

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CHAPTER 5

LEUKOCYTE ADHERENCE INHIBITION (LAI) IN RATS BEARING TRANSPLANTABLE SYNGENEIC TUMORS OF DIFFERENT IMMUNOGENICITY*

5.1 Introduction

The tube leukocyte adherence inhibition (LAI) assay has been used previously with considerable success to monitor cell mediated immunity in cancer patients (1,2). However, the assay was found to have two serious limitations. These were the quality and the durability of the tumor extracts. The assay performed under optimal conditions had a sensitivity of 62% and a specificity of around 95% in colorectal cancer patients. This sensitivity of 62% could not be improved by using extracts that had been partially purified by sephacryl S-200 column chromatography. Any further purification of the extracts was severely hindered by the limited amount of tumor material obtained at surgery.

As large amounts of tumor material are necessary in order to prepare and purify the putative LAI-reactive material, it became imperative to search for a well defined animal tumor model which could be used as a constant source of large amounts of tumor material and which could also be used to investigate and improve conditions necessary for a more sensitive and reliable tube LAI assay.

Since the tube LAI assay had been successfully used to detect immunity in rats (3-7) and since three well defined transplantable syngeneic tumors of increasing immunogenicity in rats were available, the animal studies described here were undertaken to improve the LAI assay.

5.2 Materials and Methods

5.2.1 *Rats*

Male rats of inbred BN and WAG strains were used. The animals were bred under specific-pathogen-free conditions and were 8-10 weeks old.

5.2.2 *Tumors*

5.2.2.1 Liposarcoma (LS175)

LS175 is a liposarcoma which originated as a spontaneous tumor in a female BN rat in the pancreatic, retroperitoneal region. The tumor was observed when the animal

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was 80 weeks old (8). Histopathological examination revealed that the infiltrating tumor was composed mainly of pale, swollen polyhedral cells with a granular cytoplasm containing fat. The tumor is palpable as early as one week after subcutaneous (s.c.) implantation. Immunization-challenge experiments performed according to the method of Prehn and Main (9), revealed that LS175 is not immunogenic i.e. the tumor growth is not inhibited or enhanced in immunized hosts. The tumor is transplantable in syngeneic animals.

5.2.2.2 Colon adenocarcinoma (CC531)

CC531 was induced by treatment of WAG rats with 1,2 dimethylhydrazine (DMH). It originated in the ascending colon 40 weeks after six weekly injections of 30 mg/kg DMH (10). The tumor is a moderately differentiated adenocarcinoma which is transplantable in syngeneic animals. When 2 mm cubes are implanted s.c., about 4 weeks are needed for the tumor to grow to a diameter of 10 mm. Classical immunization-challenge experiments (9) revealed that CC531 is weakly immunogenic i.e. a slight but significant tumor growth inhibition is observed in immunized hosts.

5.2.2.3 Skin basal cell carcinoma (1618)

1618 is a transplantable radiation induced basal cell carcinoma of the skin in the WAG rat (11). The tumor has strong immunogenic properties i.e. it does not grow in immunized hosts. Tumor 1618 has a doubling time of 2.5 days when implanted s.c.

5.2.2.4 Tumor implantation

Two mm cubes of tumor LS175, CC531 or 1618 were implanted s.c. in the right flank of the experimental animals. LAI assays were performed about 14 days after implantation when the tumors were either palpable (tumors CC531 and 1618) or had reached a diameter of between 5 and 10 mm (tumor LS175). LAI assays were also performed sequentially in rats bearing tumor CC531 on days 7, 14 and 21 after implantation.

5.2.2.5 Sensitization of WAG rats with irradiated 1618 tumor cells

1618 tumor cell suspensions were prepared from s.c. tumor implants. Tumor cells were isolated according to the method of Reinhold (12). Cells were washed twice with RPMI 1640 medium (GIBCO), X-irradiated at a dose of 80 GY and mixed with Freund's incomplete adjuvant. WAG rats were immunized twice at 14 days interval by intraperitoneal injections with 10^8 cells. LAI assays were performed a week after the first and 2 days after the second immunization.

5.2.3 *Preparation of tissue extracts*

Tumor tissue, obtained from s.c. implants was resected aseptically and necrotic parts were removed. Tissue extracts were prepared using the same technique that was used for the preparation of crude extracts of human tumors (3.2.2). Extracts of kidneys from normal WAG and BN rats were prepared in the same manner.

5.2.4 *Isolation of peripheral blood leukocytes (PBL)*

Peripheral blood from normal rats and rats with progressively growing tumor was drawn under ether anesthesia. Two to three ml of blood were collected from the tail vein into heparinized tubes. PBL were isolated by gradient centrifugation (13). Briefly, blood was diluted 1:1 with RPMI 1640 medium, layered over lymphocyte separation medium (LSM, Litton Bionetics) and centrifuged for 20 minutes at 500 G. Cells at the plasma/LSM interface were collected and washed twice in RPMI medium. After counting and viability testing using trypan blue dye exclusion, the suspension was adjusted to a concentration of 5×10^6 living cells per ml.

5.2.5 *Tube LAI assay*

The LAI assay was performed in triplicate in 12.5 ml glass round bottom tubes as described previously (2.2.4). The assay was terminated either after 2 hours or 20 hours. The results were expressed as nonadherent index (NAI). The NAI values outside the limits of mean $\pm$ twice the standard deviation of the control group were considered to represent a significant effect.

5.3. **Results**

When PBL from non-immunized controls or tumor-bearing rats were incubated in test tubes without the addition of tumor extract for either 2 hours or 20 hours, between 10% and 15% of the cells remained nonadherent. The addition of non-specific tumor extract resulted in an increase in the non-specific inhibition of adherence which was proportional to the protein concentration. Table 1 shows the representative results of a dose effect study performed using the PBL of rats bearing LS175 tumor with 1618 tumor extract.

Table 1. Percentage nonadherent PBL per tube LAI at various crude tumor antigen concentrations.

Concentration of crude 1618 antigen extract in μg per tube	Percent nonadherence	
	LS175 tumor-bearers	normal rats
400	58	58
300	54	65
200	46	61
100	40	47
50	22	23
—	10	15

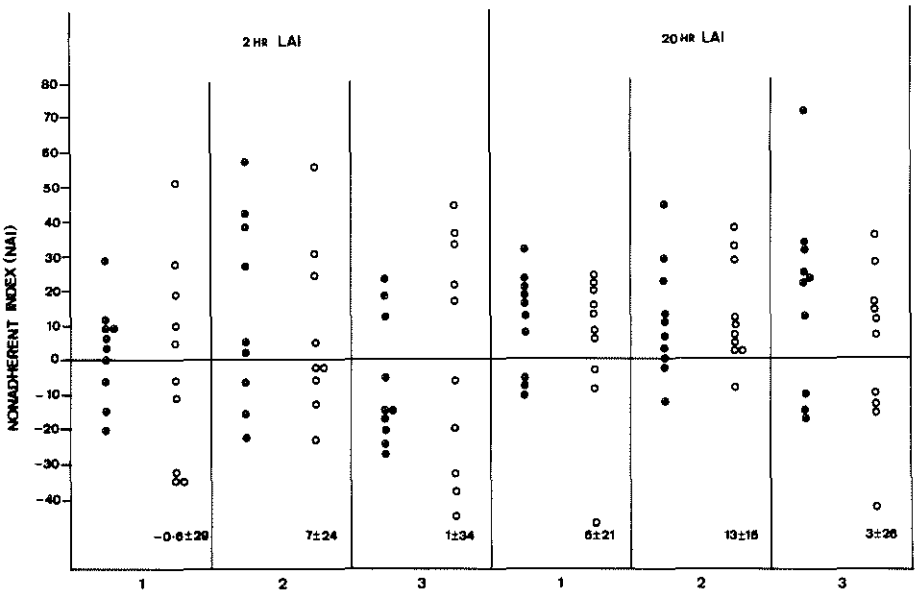

Figure 1. Distribution of NAI values in tumor-bearing BN rats (•) and control BN rats (○). The LAI assays were performed 14 days after s.c. implantation of tumor LS175. Extract of tumor LS175 served as *specific antigen* and extracts of normal BN kidneys (1), tumor CC531 (2) and tumor 1618 (3) as *non-specific antigens*. The NAI values outside the limits of mean ± twice the standard deviation of the control group were considered to represent a significant effect.

Fig. 1 shows the LAI results obtained in the initial studies with the non-immunogenic LS175 tumor-bearing BN rats. The LAI assay was performed using LS175 tumor extract as the specific antigen and extracts of normal kidney tissue and tumors CC531 and 1618 as non-specific antigens. The results were expressed as NAI. The NAI values outside the limits of mean ± twice the standard deviation of the control group were considered to represent a significant effect. The results of the 2 hours LAI assay show that using kidney extract or 1618 extract as non-specific antigen none of the 10 tumor-bearing animals showed any positive LAI-reactivity (lanes 1 and 3 respectively). When CC531 extract was used as non-specific antigen, one tumor-bearer showed a positive LAI-reactivity (lane 2). Increasing the LAI incubation time to 20 hours resulted in no significant improvement in the LAI-reactivity. Using CC531 and 1618 extracts as non-specific antigens, only one animal in each group showed a positive LAI-reactivity (lanes 2 and 3 respectively).

The results of the studies using the weakly immunogenic tumor CC531 are shown in Fig. 2. The LAI assays were performed using the same conditions as those described for LS175 tumor-bearing animals. Extract of CC531 was used as the specific antigen and extracts of normal kidneys, LS175 and 1618 as the non-specific antigens. The results show that positive LAI-reactivity was observed in one out of

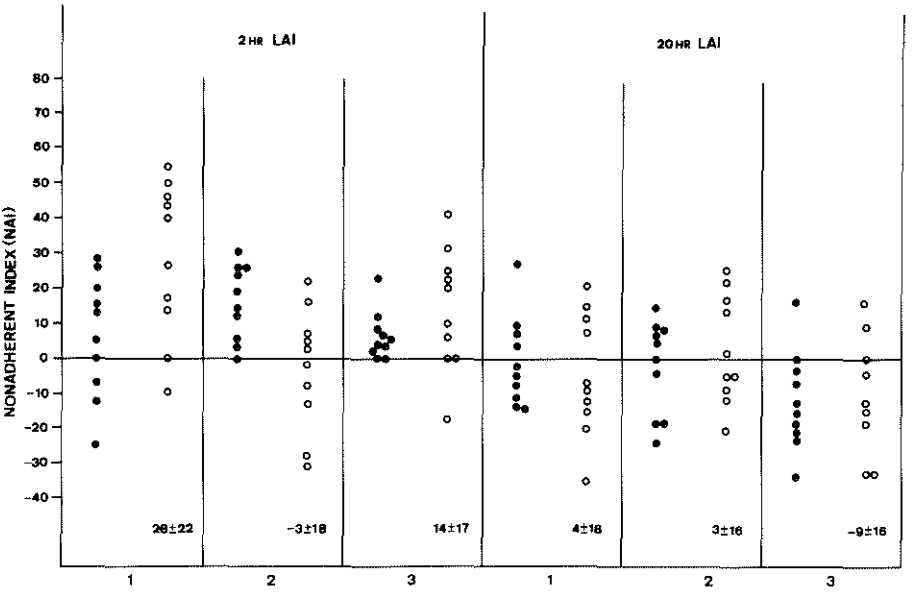

Figure 2. Distribution of the NAI values in tumor-bearing WAG rats (●) and control WAG rats (○). The LAI assays were performed 14 days after s.c. implantation of tumor CC531. Extract of tumor CC531 served as *specific antigen* and extracts of normal WAG kidneys (1), tumor LS175 (2) and tumor 1618 (3) as *non-specific antigens*. The NAI values outside the limits of mean ± twice the standard deviation of the control group were considered to represent a significant effect.

ten tumor-bearers when kidney extract was used as non-specific antigen (lane 1). In the 20 hours LAI assay, none of the ten tumor-bearers showed any positive LAI-reactivity. In a separate series of experiments in which WAG rats were sequentially monitored using 2 hours LAI assay on days 7, 14 and 21 after tumor implantation, positive LAI-reactions were occasionally observed at day 14 in one or two animals from a group of ten. However, when tested on day 21, these animals did not show positive LAI-reactivity.

Fig. 3 shows the results obtained in WAG rats bearing the highly immunogenic 1618 tumor. In the 2 hours LAI assay, when 1618 extract is used as specific antigen and extracts of LS175 and CC531 as non-specific antigens, one out of 10 tumor-bearers in each group showed a positive LAI-reactivity (lanes 2 and 3 respectively). One tumor-bearer showed a positive LAI-reactivity in the 20 hours LAI assay when 1618 extract was used as specific antigen and LS175 extract was used as non-specific antigen (lane 2). Similar results were also observed using both the LAI assays in WAG rats which had been immunized with 1618 tumor cell suspensions. None of the ten immunized animals showed any positive LAI-reactivity either a week after the first immunization or 2 days after the second immunization.

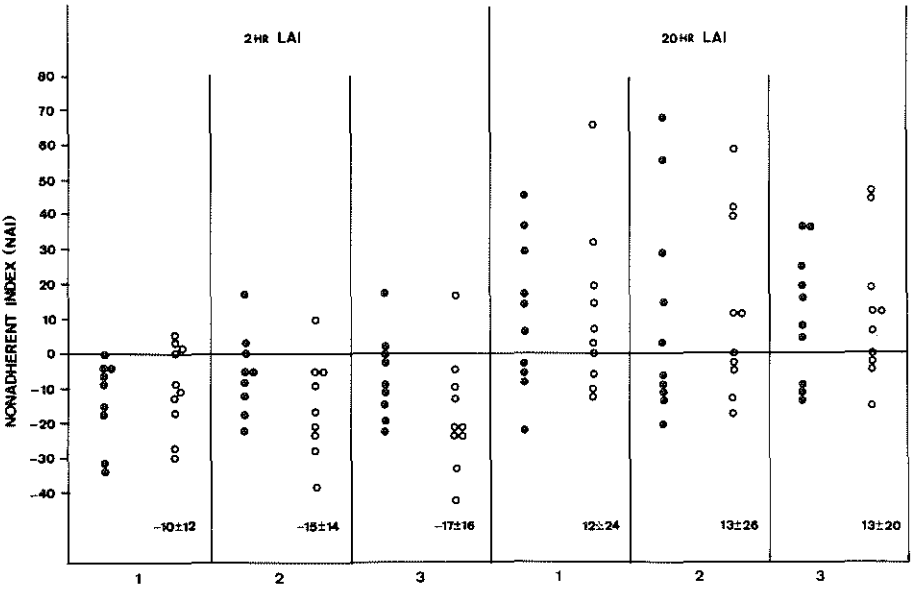

Figure 3. Distribution of the NAI values in tumor-bearing WAG rats (●) and control WAG rats (○). The LAI assays were performed 14 days after s.c. implantation of tumor 1618. Extract of tumor 1618 served as *specific antigen* and extracts of normal WAG kidneys (1), tumor LS175 (2) and tumor CC531 (3) as *non-specific antigens* . The NAI values outside the limits of mean ± twice the standard deviation of the control group were considered to represent a significant effect.

5.4 Discussion

In the present studies, the 2 hours tube LAI assay which was successfully used in humans (1,2) has been used to follow the LAI-reactivity of the peripheral blood leukocytes (PBL) of rats bearing transplantable syngeneic tumors. The initial LAI studies were performed in BN rats bearing a transplantable syngeneic LS175 tumor. The results obtained show that using the conventional 2 hours tube LAI assay, sporadic tumor-specific LAI-reactivity was observed. In previous studies in rats bearing syngeneic tumors, Kalafut et al. (4) and Hung et al. (5) observed that prolongation of the LAI assay incubation time to 20 hours gave better and consistent results than the 2 hours conventional assay. We therefore decided to use in addition, the 20 hours tube LAI assay. Once again, only sporadic tumor-specific LAI-reactivity was observed in the PBL of tumor-bearers. There was no qualitative difference between the results of the two LAI assays used. The possible explanation for this lack of significant tumor-specific LAI-reactivity could be that the LS175 tumor which is non-immunogenic does not or only weakly evokes an immune response which is occasionally detected in the tube LAI assay.

To investigate this possibility, LAI studies were performed using PBL of WAG rats bearing the weakly immunogenic CC531 tumor. Sporadic tumor-specific LAI-reactivity was observed using both the LAI assays. Occasional positive LAI-reactions were noted when CC531 implanted rats were sequentially monitored on days 7, 14 and 21. The results obtained using the highly immunogenic tumor 1618 were similar to those obtained with the non-immunogenic and the weakly immunogenic tumors. The results of studies using WAG rats immunized with cell suspensions of tumor 1618 also failed to demonstrate a consistent tumor-specific LAI-reactivity.

The results presented here contradict those obtained earlier by Kalafut et al. (4), Hung et al. (5) and those reported recently by Morizane and Sjögren (6,7). All these authors were able to demonstrate significant tumor-specific LAI-reactivity in PBL of tumor-bearing rats. In the studies by Kalafut et al. (4) and Hung et al. (5) the 20 hours LAI assay was performed using homologous serum and fetal calf serum (FCS)-supplemented media, respectively. FCS-supplemented medium was also used in the micro-glass-tube LAI assay by Morizane and Sjögren (6). In the current studies, when the tube LAI assays were performed using FCS-supplemented medium, no tumor-specific LAI-reactions were observed with the PBL of the rats bearing LS175, CC531 and 1618 tumors. A FCS concentration of 1% in the assay system totally inhibited the adherence of PBL from both normal and tumor-bearing rats. Similar observations were reported previously (3,14,15). In those studies, normal serum or FCS inhibited the adherence of normal and "immune" leukocytes and abolished the specific LAI-reactions in the tube LAI assay.

The failure to observe any significant tumor-specific LAI-reaction in this study could be due to the following reasons. Firstly, a lack of cells in the PBL that mediate the specific LAI-reaction. It has been shown previously that in the tube LAI assay, cells of the monocyte/macrophage series play a central role in triggering the cascade of events leading to nonadherence (16). Peritoneal cell suspensions (PC) contain mainly cells of the monocyte/macrophage series. In previous studies Holan et al. (17) observed specific LAI-reactions in PC of rats immunized with various antigens. However, when we performed pilot studies with the PC of LS175, CC531 and 1618 tumor-bearing rats, no tumor-specific LAI-reactions were observed.

The sporadic tumor-specific LAI-reactivity in this study could also have been due to an insufficient amount of LAI-reactive tumor antigen in the crude tumor extracts. Increasing the concentration of these antigens by further purification of the crude extracts could possibly lead to a higher number of specific-reactions. However, the possibility that the LAI-reactive material is lost during the early stages of the preparation of the extracts has to be taken into consideration.

Finally it is possible that the site of tumor implantation in the current studies was inappropriate to evoke a specific LAI response. However, it is strange that the same tumor implantation site in the immunization-challenge experiments resulted in the tumor-specific immunity (CC531 and 1618 tumors). The unlikely possibility remains that PBL are poor effectors of tumor-associated immunity of s.c. implanted tumors. Perhaps the leukocytes isolated from regional draining lymph

nodes would be more suitable as tube LAI effector cells (18). Transplantable syngeneic tumors may also induce suppressor cells or factors which impair the immune response of the host (19). Therefore, it could be assumed that consistent tumor-specific LAI-reactions can be successfully detected only in animals with a primary autochthonous tumor.

5.5 References

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CHAPTER 6

LEUKOCYTE ADHERENCE INHIBITION (LAI) IN RATS USING PARTIALLY PURIFIED TUMOR EXTRACTS*

6.1 Introduction

The sporadic tumor-specific leukocyte adherence inhibiton (LAI)-reactivity in rats bearing non-immunogenic liposarcoma (LS175), weakly immunogenic colon adenocarcinoma (CC531) and highly immunogenic skin basal cell carcinoma (1618) tumors as presented in the preceeding chapter could have been due to several factors (1).

In the studies described here, close attention was focused on one of these factors. The possibility that the sporadic LAI-reactivity in the tumor-bearers was caused by an insufficient amount of LAI-reactive tumor antigen in the crude extracts that were used was investigated further. It was assumed that a higher number of tumor-specific LAI-reactions might be observed, if the concentration of the LAI-reactive antigens could be increased by further purification of the crude extracts.

WAG rats bearing the weakly immunogenic colon adenocarcinoma (CC531) tumor model was chosen since successful results were obtained in colorectal cancer patients using extracts that had been obtained after column chromatography (chapter 4).

Tube LAI assay was performed using peripheral blood leukocytes (PBL) and peritoneal cells (PC) of CC531 tumor-bearing animals. Crude tumor extracts were partially purified using sephacryl S-200 column chromatography. To exclude the possibility that the LAI-reactive material could have been lost during the early stages of preparation, LAI studies were also performed with extracts that had been sequentially isolated.

6.2 Materials and Methods

6.2.1 *Rats*

Male rats of inbred BN and WAG strains were used. The animals were bred under specific-pathogen-free conditions and were 8-10 weeks old.

*This chapter has been submitted for publication.

6.2.2 *Tumors*

6.2.2.1 Liposarcoma (LS175)

LS175 is a liposarcoma which originated as a spontaneous tumor in a female BN rat in the pancreatic, retroperitoneal region (2). Immunization-challenge experiments performed according to the method of Prehn and Main (3), revealed that LS175 is not immunogenic, i.e. the tumor growth is not inhibited or enhanced in immunized hosts. The tumor is transplantable in syngeneic animals.

6.2.2.2 Colon adenocarcinoma (CC531)

CC531 was induced by treatment of WAG rats with 1,2 dimethylhydrazine (DMH). It originated in the ascending colon 40 weeks after six weekly injections of 30 mg/kg DMH (4). The tumor is a moderately differentiated adenocarcinoma which is transplantable in syngeneic animals. When 2 mm cubes are implanted s.c., about 4 weeks are needed for the tumor to grow to a diameter of 10 mm. Classical immunization-challenge experiments (3) revealed that CC531 is weakly immunogenic, i.e. a slight but significant tumor growth inhibition is observed in immunized hosts.

6.2.2.3 Tumor implantation

Two mm cubes of tumor LS175 or CC531 were implanted s.c. in the right flank of BN and WAG rats, respectively. LAI assays were performed 14 days after implantation when the tumor CC531 was palpable. BN tumor-bearers served only as a source of LS175 tumor material.

6.2.3 *Preparation of tissue extracts*

Tumor tissue, obtained from s.c. implants was freed of necrotic, fatty and fibrous tissues. The specimen was then cut into small pieces and a 20% (w/v) homogenate was prepared in ice-cold phosphate buffered saline (PBS) using an ultra-turrax TR 18-10 homogenizer. The homogenate was centrifuged for 10 minutes at 1000G. The supernatant was divided into 3 equal parts. The first part was left untreated and referred to as 1000G extract. The second part was centrifuged at 20000G for another 60 minutes. The supernatant was referred to as 20000G extract. The third part was centrifuged at 50000G for 60 minutes. The supernatant was divided into two parts. One part was left untreated and referred to as 50000G extract. The second part was subjected to column chromatography after it had been concentrated four fold using a minicon concentrator B15 (Amicon). Extracts of colon, kidneys and liver from normal WAG rats were prepared in the same manner. The protein concentration of the stock extracts was determined using the Bio-rad protein assay (Bio-rad, Holland) and ranged from 3 to 7 mg/ml.

All extracts were sterilized by filtration through 0.2μ millipore filter. They were stored at –70°C in 1 ml aliquots and were used only once.

6.2.4 *Sephacryl S-200 column chromatography*

Sephacryl S-200 column chromatography was performed using the same conditions as those described for the human tumor extracts (4.2.3). All LAI studies were performed using "F 120" (partially purified extract) since it was demonstrated previously in human studies that most LAI-reactivity was present in the high molecular weight fraction (Chapter 4). Similar observations were previously reported by Thomson et al. (5).

6.2.5 *Isolation of peripheral blood leukocytes (PBL)*

PBL were isolated from heparinized blood as described under 5.2.4.

6.2.6 *Peritoneal cells*

Peritoneal cells were collected from normal and tumor-bearing rats. Rats were anaesthetized with Nembutal (Abbott Laboratories) and injected intraperitoneally with 50 ml RPMI 1640 supplemented with 10% fetal calf serum (FCS). The solution was withdrawn and collected in plastic tubes. The tubes were centrifuged at 800G for 10 minutes. The pellets were washed twice with RPMI 1640 without FCS. After viability testing, the cell suspension was adjusted to a concentration of 5×10^6 living cells per ml. Suspensions containing more than 20% erythrocytes were discarded.

6.2.7 *Tube LAI assay*

The LAI assay was performed in triplicate in 12.5 ml glass round bottom tubes as described previously (2.2.4). Each tube contained 0.3 ml RPMI medium, 0.1 ml of the leukocyte suspension (5×10^5 cells), and 0.1 ml (100 μg protein) of the partially purified or sequentially isolated tumor extract. The assay was terminated after 2 hours. The results were expressed as nonadherent index (NAI). The NAI values outside the limits of mean $\pm$ twice the standard deviation of the control group were considered to represent a significant effect.

6.3 **Results**

Figure 1 shows the LAI results obtained with the PBL of WAG rats bearing CC531 tumor. The LAI assay was performed using partially purified CC531 tumor extract as the specific antigen and partially purified extracts of tumor LS175, normal colon,

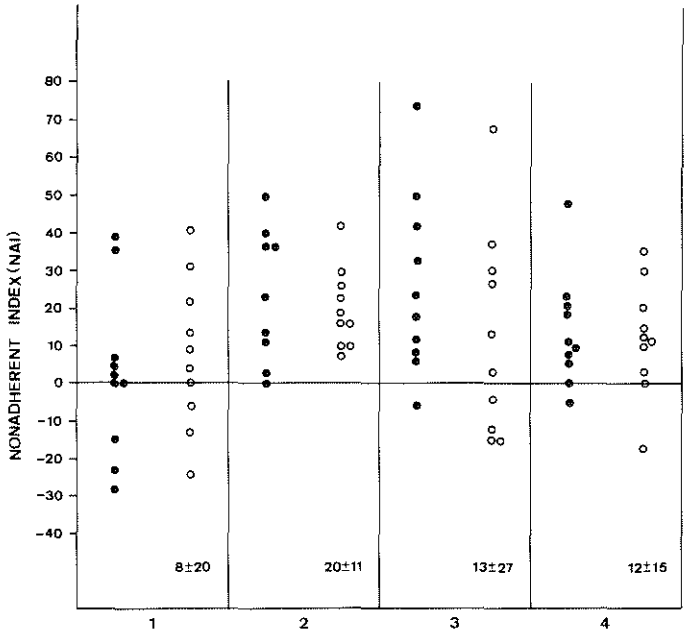

Figure 1. Distribution of the NAI values in tumor-bearing WAG rats (●) and control WAG rats (○). The LAI assay was performed 14 days after s.c. implantation of tumor CC531. Partially purified extract of tumor CC531 served as *specific antigen* and partially purified extracts of tumor LS175 (1), normal colon (2), normal kidneys (3), normal liver (4) as *non-specific antigens.* The NAI values outside the limits of mean ± twice the standard deviation of the control group were considered to represent a significant effect.

kidneys and liver as non-specific antigens. The results were expressed as NAI. The NAI values outside the limits of mean ± twice the standard deviation of the control group were considered to represent a significant effect. The results show that using LS175 extract as non-specific antigen, none of the 10 tumor-bearing animals showed any positive LAI-reactivity (lane 1). When normal colon, kidneys and liver extracts were used as non-specific antigens, one animal in each group showed a positive LAI-reactivity (lanes 2, 3 and 4, respectively). Similar results were also observed when PC were used instead of the PBL.

The results of the studies using the sequentially isolated extracts of tumor CC531 and normal kidneys are shown in Figure 2. The LAI assay was performed using PBL of CC531 tumor-bearing animals. Extracts of CC531 (1000G, 20000G, 50000G and partially purified) were used as the specific antigen and the corresponding extracts of normal kidneys (1000G, 20000G, 50000G and partially purified) were used as the non-specific antigens. The results show that positive LAI-reactivity was observed in one out of ten tumor-bearers when 50000G kidney extract was used as non-specific antigen (lane 3).

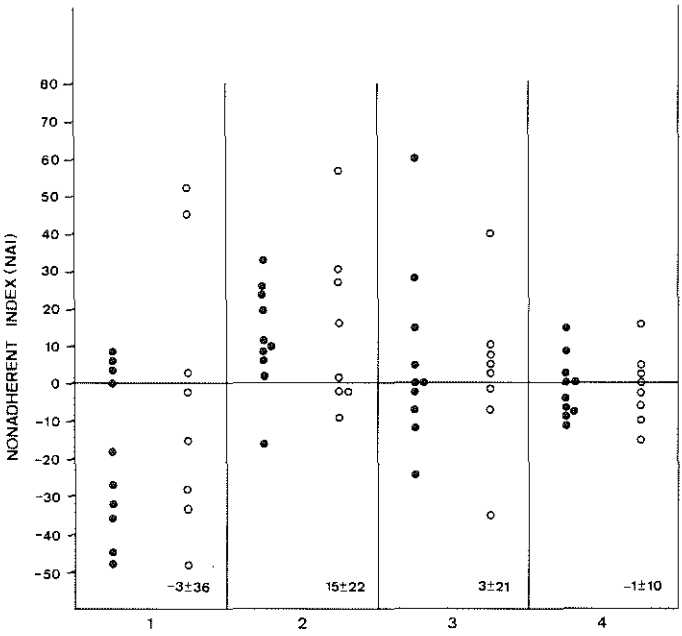

Figure 2. Distribution of the NAI values in tumor-bearing WAG rats (●) and control WAG rats (○). The LAI assay was performed 14 days after s.c. implantation of tumor CC531. Extracts of tumor CC531 (1000G, 20000G, 50000G and partially purified) served as *specific antigen* and corresponding extracts of normal WAG kidneys; 1000G extract (1), 20000G extract (2), 50000G extract (3) and partially purified extract (4) as *non-specific antigens.* The NAI values outside the limits of mean ± twice the standard deviation of the control group were considered to represent a significant effect.

Table 1. A comparison of means of percentage nonadherence in control rats and rats bearing CC531 tumor.

Type of extract	Mean nonadherence in percent	
	control animals (n=20)	CC531 tumor-bearers (n=20)
RPMI 1640 medium	23 ± 5%	22 ± 6%
Normal kidney 1000G extract	71 ± 15%	66 ± 16%
Normal kidney 20000G extract	55 ± 11%	56 ± 17%
Normal kidney 50000G extract	57 ± 11%	53 ± 15%
Normal kidney sephacryl S-200 partially purified extract	46 ± 10%	44 ± 9%
Tumor CC531 1000G extract	68 ± 11%	65 ± 20%
Tumor CC531 20000G extract	70 ± 10%	69 ± 14%
Tumor CC531 50000G extract	68 ± 14%	65 ± 14%
Tumor CC531 sephacryl S-200 partially purified extract	45 ± 11%	42 ± 7%

LAI assays were performed using sequentially isolated and partially purified extracts. There are no significant differences between the two groups irrespective of the type of extract used.

Table 1 shows a comparison of means of percent nonadherence in 20 CC531 tumor-bearers and 20 control animals. The LAI assay was performed using PBL and extracts of CC531 (1000G, 20000G, 50000G and partially purified) as specific antigen and corresponding extracts of normal kidneys as non-specific antigen. The results show that there are no significant differences in the means of percentage nonadherence between CC531 tumor-bearers and control animals regardless of the type of extract that was used.

6.4 Discussion

In our previous studies (1), the conventional tube LAI assay which proved successful in humans, was used to follow the LAI-reactivity of peripheral blood leukocytes (PBL) of rats bearing transplantable syngeneic tumors of different immunogenicity. An absence of any significant tumor-specific LAI response in that study could have been due to a number of factors. The most likely factor could have been the insufficient amount of LAI-reactive tumor-associated antigen(s) in the crude extracts that were used.

In the current study, this possibility has been investigated further using the weakly immunogenic, CC531 tumor-bearing animals. The choice of this tumor model for further investigation was based on the fact that significant tumor-specific LAI responses were observed in colon cancer patients using partially purified tumor extracts.

The results of the LAI studies performed using the partially purified CC531 extract failed to demonstrate any significant tumor-specific LAI-reactivity in the PBL of CC531 tumor-bearers. This could have been due to a lack of cells in the PBL that mediate the specific LAI-reaction. It has been reported earlier that in the tube LAI assay, cells of the monocyte/macrophage series are central in triggering the cascade of events leading to nonadherence (6). However, when peritoneal cell suspensions (PC) which contain mainly cells of the monocyte/macrophage series, were used instead of the PBL, sporadic LAI-reactivity was once again observed. A likely explanation for these results could be that instead of concentrating the LAI-reactive material by partial purification of the crude extracts, a significant proportion of the LAI-reactive material was lost during the initial preparation and sephacryl S-200 column chromatography of the tumor extract.

Therefore, additional LAI studies were performed using tumor extracts that had been sequentially isolated. The results showed that irrespective of the stage of isolation of the antigen extract, only sporadic positive LAI response was noted in CC531 tumor-bearers. It is possible that since the LAI-reactivity is expressed in the form of nonadherent index (NAI), a small difference in the reactivity with the specific and non-specific extract remains indistinguishable. It would perhaps be preferable to compare the means of nonadherence in the tumor-bearers and control animals in the presence of various extracts. When such a comparison was made, it was observed that there were no significant differences in the means of non-adherence between CC531 tumor-bearers and the control animals irrespective of

the type of extract used. These results seem to indicate that the sporadic LAI-responses observed in our previous study (1) could have been due to a total absence of LAI-reactive material rather than its presence in low concentration in the crude tumor extract that were used.

An alternative explanation for the absence of any consistent tumor-specific LAI-reactivity in this study could be that the site of implantation of the CC531 tumor was unsuitable to educe any specific LAI response. However, the same site of CC531 tumor implantation resulted in a tumor-specific immunity when immunization-challenge experiments were performed. It is known that transplantable syngeneic tumors may also induce suppressor cells or factors which inhibit the immune response of the host (7). It can therefore be assumed that consistent tumor-specific LAI-reactions can be successfully detected only in animals with a primary autochtonous tumor.

In conclusion, the results reported here indicate that consistent tumor-specific LAI-reactions (if any) in rats bearing a syngeneic transplantable tumor cannot be observed in the conventional tube LAI assay. Our original goal to use the rat model for parallel studies to improve the human tube LAI assay therefore remains unachieved.

6.5 References

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CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

The various types of LAI assay fall into one of the three general categories: the hemocytometer LAI (1), the microplate LAI (2) and the tube LAI (3) methods. The LAI assay measures the ability of sensitized human and animal leukocytes to recognize and react with the appropriate tumor extract(s) (and other antigens) resulting in the release of soluble immunopharmacological mediator(s) which reduce leukocyte adherence to glass or plastic surfaces.

A number of investigators have used the LAI assay in the clinical setting for several years and have found it to be reproducible, specific to tumor extract type and a reliable in vitro monitor of cell mediated immunity (CMI) (4-8). On the other hand, some investigators have found the LAI test unreliable and of little or no clinical value (9-11).

Although, the three categories of the LAI assays have different mechanisms, they are methodologically simpler and quicker than other methods for the detection of CMI (12-16). At the same time, however, there is general consensus amongst investigators who use the LAI test that, in order to obtain consistent results, it is imperative that the LAI assay be performed routinely and by skilled laboratory personnel. The individual merits of the three categories of the LAI assay, their individual mechanisms and their applications have been extensively reviewed elsewhere (17,18).

7.2 Tube LAI assay in patients

The goal of the initial studies described in chapter 2 was to confirm that the tube LAI assay was indeed a reliable assay and could be used for the detection of tumor-associated immunity in cancer patients. Peripheral blood leukocytes (PBL) of 70% of both breast and gastrointestinal cancer patients reacted positively with the relevant allogeneic tumor extracts when tested in this assay. This 70% sensitivity of the assay is lower than that observed by others (8,19) who reported a sensitivity of 80-95%. With regards to the specificity of the assay, 13% of the control individuals reacted positively. This represents a specificity of 87% which is lower than that observed by others (20,21) who reported a value of 95%. Although, neither the sensitivity nor the specificity of the assay was high, it was concluded that the assay was a useful tool for investigating tumor immunity in cancer patients.

The two main drawbacks (low sensitivity and limited "shelf-life" of the crude extracts) of the tube LAI assay formed the focus point of the studies described in

chapter 3. During the initial studies mentioned in chapter 2, it was observed that extensive prescreening of the crude tumor extracts was imperative in order to select extracts that discriminated well in the assay and that well discriminating extracts had a limited "shelf-life" and thus could not be used beyond a period of 4 months. Therefore, LAI assays were performed using tumor extracts containing minimal sub-cellular impurities in order to assess whether such extracts increased the sensitivity of the LAI assay and at the same time had an improved "shelf-life". Out of a total of 49 extracts that were prepared, 40 were usable for a period not exceeding 4 months. The "shelf-life" of these extracts was thus similar to that observed in the initial studies (chapter 2). The assay was found to have a sensitivity of 62% when patients with colorectal cancer were tested. It had a specificity of 95% in the two groups of patients with non-malignant disorders of the colon. It was therefore evident from these results, that although neither the sensitivity of the assay, nor the "shelf-life" of the extracts was improved, the high number of false positive reactions observed in chapter 2 were reduced from 13% (specificity of 87%) to an acceptable level of 5% (specificity of 95%) by the use of extracts with minimal sub-cellular impurities.

This reduction in the number of false positive reactions provided the objective for the investigations described in chapter 4, where an attempt was made to increase the sensitivity of the assay while retaining its high specificity by using tumor extracts that had been partially purified by sephacryl S-200 column chromatography. It was also examined whether such partially purified extracts showed an extended "shelf-life" as compared to their crude counterparts. Seven out of eleven colorectal cancer patients (64%) reacted positively when partially purified extracts were used. Thus, there was a slight but insignificant increase in the sensitivity of the LAI assay while the 95% specificity was retained. Five percent of the patients suffering from non-gastrointestinal diseases reacted positively with the partially purified colorectal cancer extracts. Out of a total of 24 extracts that were partially purified, most of them were used up within 3 months after preparation due to their low protein concentration. Only 2 partially purified extracts could be tested beyond 3 months and were found to be unreactive within 4 months suggesting that the partial purification did not lead to an extended "shelf-life". When colorectal cancer patients were tested simultaneously with crude colorectal cancer extracts and their partially purified counterparts, 50% of the patients reacted positively in both instances suggesting that the partial purification of the crude extracts did not increase the sensitivity of the assay. Similar observations were reported previously by others (22). The low protein concentration of the partially purified extracts placed severe restrictions on the number of patients that could be tested. The amount of patient tumor material also limited the amounts of crude extracts that could be chromatographed and purified further using other physico-chemical methods.

7.3 Tube LAI in rats

In order to persue the initial objectives of increasing the sensitivity of the LAI assay and to improve the durability of the tumor extracts, large amounts of tumor material were necessary for the preparation and purification of the putative LAI-reactive material. Therefore, it became imperative to search for a continuous source of large amounts of tumor material. One such source would be a suitable animal tumor model which could not only act as a continuous source of tumor material but could also be used to investigate and improve conditions necessary for a more sensitive and reliable tube LAI assay.

Since the tube LAI assay was previously used to detect tumor immunity in rats (23,24) and since three well defined transplantable syngeneic tumors of increasing immunogenicity in rats were available, the studies described in chapter 5 were undertaken. The initial LAI studies were performed using PBL of BN rats bearing a transplantable syngeneic liposarcoma LS175 tumor which is non-immunogenic. Using the PBL and crude tumor extracts in the conventional 2 hours tube LAI assay, only sporadic tumor-specific LAI-reactivity was observed. It was previously reported by Hung et al. (23) and Kalafut et al. (25), that prolongation of the assay incubation time to 20 hours gave more consistent LAI-reactivity. However, when the assay time in the present studies was increased to 20 hours, only sporadic tumor-specific LAI- reactivity was observed. There was no qualitative difference between the results of the two LAI assays. One possible explanation for this lack of significant tumor-specific LAI-reactivity could be that the LS175 tumor which is non-immunogenic does not or only weakly evokes an immune response which is occasionally detected in the tube LAI assay. This possibility was investigated using WAG rats bearing CC531 colon tumor which is weakly immunogenic and skin tumor 1618 which is highly immunogenic. In both these tumor models, only sporadic tumor-specific LAI-reactivity was observed. The results of studies in which WAG rats were immunized with cell suspensions of highly immunogenic tumor 1618 also failed to demonstrate a consistent tumor-specific LAI-reactivity. These results contradict those obtained earlier by Hung et al. (23) and Kalafut et al. (25) and recently by Morizane and Sjögren (24). All these authors observed a significant tumor-specific LAI-reactivity in PBL of rats bearing immunogenic tumors. Neither the inclusion of fetal calf serum (FCS) in the LAI assay system nor the use of peritoneal cells instead of PBL resulted in any consistent LAI-reactivity in any of the three tumor models that were investigated. Two possible explanations are: a) that the site of tumor implantation was inappropriate to evoke a specific LAI response. This is unlikely, since the same tumor implantation site in the immunization-challenge experiments resulted in the tumor-specific immunity. b) that insufficient amount of LAI-reactive material was present in the crude extracts. Increasing the concentration of the LAI-reactive material by further purification of the crude extracts could then lead to a more consistent LAI-reactivity.

This possibility was investigated in the studies described in chapter 6. Tube LAI assays were performed using PBL of the weakly immunogenic CC531 tumor-bearing animals and tumor extracts that were partially purified by sephacryl S-200

column chromatography. No significant tumor-specific LAI-reactivity was observed. Sporadic LAI-reactivity was also observed when peritoneal cells were used instead of the PBL. An explanation for this lack of consistent tumor-specific LAI-reactivity could be that instead of concentrating the LAI-reactive material by partial purification of the crude extracts, a significant proportion of the LAI-reactive material was lost during the initial preparation and sephacryl S-200 column chromatography of the tumor extracts. Thus, additional studies were performed using tumor extracts that had been sequentially isolated. Once again, irrespective of the stage of isolation of the antigen extract, only sporadic LAI response was observed. When the means of the percentage nonadherence in the CC531 tumor-bearers and control animals using various sequentially isolated tumor extracts were compared, no significant differences were observed. Therefore, it could be concluded that the sporadic LAI responses observed in the studies described in chapter 5 could have been due to a total lack of LAI-reactive material rather than its presence in low concentration in the crude tumor extracts. The unlikely possibility remains that both PBL and peritoneal cells are poor effectors of tumor-associated immunity of subcutaneously implanted tumors. Perhaps the leukocytes isolated from regional draining lymph nodes would be more suitable as tube LAI effectors. Transplantable syngeneic tumors may also induce suppressor cells or factors which inhibit the immune response of the host (26). It is therefore plausible that consistent tumor-specific LAI-reactions can be successfully detected only in animals with a primary autochthonous tumor.

7.4 Concluding remarks

The effectiveness of the tube LAI assay for investigating tumor-specific immunity in cancer patients is limited by two main factors. Firstly, the quality and limited "shelf-life" of the stimulating antigen within crude tumor extracts and secondly, the limited sensitivity of the tube assay of about 70%. Despite these two limitations, the tube LAI assay can be used with success provided it is performed routinely by skilled and dedicated laboratory personnel. The partial purification of the crude extracts neither increased the "shelf-life" of these extracts nor the sensitivity of this assay. Perhaps the use of antigen preparations of a consistent quality which can be isolated from in vitro tumor cell lines may increase the sensitivity of the assay. For the present, even though this limited sensitivity excludes it from being used on its own in the clinic for monitoring cancer patients for recurrent disease or micrometastases, the sensitivity of the tube LAI assay is higher than that of other in vitro methods for the detection of cell-mediated immunity (1.3.7). One reason for this could be, that at present, although the precise nature of the LAI stimulating antigen(s) is not known, organ specific neoantigens (OSN) are the triggers of the LAI-reaction. This idea is supported by the fact that, although most spontaneous tumors are heterogeneous with numerous variations in their antigenic make-up, some common OSN determinant is preserved in the tumors of the same histological

origin. This common determinant present in all the allogeneic extracts of a given type of tumor is recognized by the effector cells with a greater consistency in the tube LAI assay than in the other assays in which the common determinant is recognized either with a lower frequency or not at all.

The clinical value of the LAI assay lies in its effectiveness in combination with other tests. This has been shown by Payne et al. (27), who using the tube LAI assay and carcinoembryonic antigen determinations, diagnosed colorectal carcinoma with 91% sensitivity. Ayeni et al. (28) also reported the tube LAI assay to be of considerable value in combination with standard physical methods for the evaluation of suspected cases, especially early ones, of colorectal cancer. The tube assay has also been used with success to monitor the partial purification of tumor-associated antigens (TAA) from crude tumor extracts (this study) and from tumor cell lines and spent tissue culture media of these cell lines (D.M.P. Thomson, personal communication).

The failure to observe any consistent tumor-specific LAI responses in the three rat tumor models used, indicate that consistent tumor-specific LAI-reactions (if any) in rats bearing transplantable syngeneic tumors cannot be observed in the conventional tube LAI assay. It may well be that while there is tumor antigen recognition by the effectors (PBL or peritoneal cells), the soluble immunopharmacological agents that mediate the LAI are not released. Whatever the reason, the original goal to use the rat model for parallel studies to improve the human tube LAI assay was not achieved.

7.5 References

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CHAPTER 8

SUMMARY

In the first half of chapter 1, some historical facts about tumor immunology are briefly considered and the general methods for the detection of tumor immunity are described. One method in particular, the leukocyte adherence inhibition (LAI) assay takes up the second half of this chapter. The LAI assay is based on the phenomenon that leukocytes from cancer patients lose their ability to adhere to glass or plastic surfaces when incubated with extracts of cancer of the same organ and histogenesis. The development of the LAI assay, its different variations (the hemocytometer LAI, the microplate LAI, the tube LAI), their individual mechanisms and their applications are reviewed in details.

In chapter 2, the applicability of one particular variation of the LAI, namely the tube LAI assay was investigated in breast and gastrointestinal cancer patients. The assay was found to detect tumor type-specific immunity in these patients with a sensitivity of around 70%. The specificity of the assay was around 87%. In order to obtain consistent results, it was necessary to perform the assay routinely. Since well discriminating tumor extracts had a limited "shelf-life", it was imperative to replace them with usable new ones.

The investigations that were persued in an attempt to increase the sensitivity and the specificity of the tube LAI assay are described in chapters 3 and 4. The LAI assay was performed using peripheral blood leukocytes (PBL) from colorectal cancer patients and patients with non-malignant disorders of the colon. Two types of tumor extracts were used. In the studies described in chapter 3, tumor extracts that contained minimal sub-cellular impurities were used, whereas extracts that were partially purified by sephacryl S-200 column chromatography were used in the studies described in chapter 4. In both these studies, the "shelf-life" of the extracts was also assessed. The results showed that, although there was no improvement in either the sensitivity of the assay or the "shelf-life" of the extracts, the specificity of the assay was improved and maintained at 95%.

Since only a limited amount of tumor material was available from patients undergoing surgery, and since large amounts of tumor material were required for the partial purification by sephacryl S-200 column chromatography and other physico-chemical methods, it became imperative to set up parallel LAI studies in rats which would not only provide a continuous source of large amounts of tumor material, but which could also be used to investigate the conditions necessary for increasing the sensitivity of the tube LAI. In the studies described in chapter 5, sporadic tumor-specific LAI-reactions were observed using both the PBL and peritoneal cells of rats bearing the non-immunogenic liposarcoma LS175 tumor,

the weakly immunogenic colon CC531 tumor and the highly immunogenic skin 1618 tumor.

Chapter 6 is devoted to the investigations performed using PBL and peritoneal cells of CC531 tumor-bearers and tumor extracts that were partially purified on sephacryl S-200 column. The results showed no consistent tumor-specific responses. In order to exclude the possibility that the lack of consistent LAI-reactivity was due to the loss of LAI-reactive material during the initial isolation and sephacryl S-200 column chromatography, additional LAI studies were performed using extracts that were sequentially isolated. Once again, no consistent tumor-specific LAI responses were observed irrespective of the stage of isolation.

In the general discussion in chapter 7, the limitations and the clinical value of the tube LAI test is reviewed. The possible reasons for the failure of this test in the three rat tumor models are also discussed.

CHAPTER 9

SAMENVATTING

In hoofdstuk 1 wordt een kort overzicht gegeven van de ontwikkelingen in de tumor immunologie en worden de methodes beschreven die kunnen worden aangewend om tumor-geassocieerde immuniteit aan te tonen. Eén methode in het bijzonder wordt behandeld nl. de leukocyten-adherentie-remmings (inhibitie) test of LAI-test. Deze test, die het onderwerp vormt van dit proefschrift, berust op het principe dat leukocyten van kanker patiënten hun vermogen om op plastic of glas te plakken gedeeltelijk verliezen (inhibitie van adherentie) indien deze cellen in contact worden gebracht met tumor extract. Voor een specifieke LAI-reactie moet dit extract ("tumor-antigeen") afkomstig zijn van een tumor die van hetzelfde type is als de patiënt heeft die de leukocyten heeft afgestaan. De verschillende varianten van de LAI-test (reageerbuis-, telkamer- en microtiter plaat methodes) worden gedetailleerd besproken; tevens wordt ingegaan op de immunologische mechanismen die aan deze methodes ten grondslag liggen.

In hoofdstuk 2 worden een aantal experimenten met de (buis) LAI-test bij patiënten met borstkanker en gastrointestinale tumoren beschreven. Het bleek dat de LAI-test een gemiddelde gevoeligheid had van 70% terwijl de specificiteit gemiddeld 87% bedroeg. Voor het verkrijgen van deze resultaten was het nodig dat de test op routine-basis werd verricht. Bovendien bleek dat de ruwe tumorextracten slechts beperkt houdbaar waren (± 3 maanden), zodat voortdurend nieuwe extracten moesten worden geproduceerd en gescreened op hun bruikbaarheid.

In de hoofdstukken 3 en 4 worden de experimenten besproken die tot doel hadden de gevoeligheid en specificiteit van de LAI-test te vergroten. De test werd uitgevoerd bij patiënten met colonkanker en patiënten met niet-kwaadaardige aandoeningen van het maagdarmkanaal. Indien gebruik werd gemaakt van tumorextracten die voornamelijk bestonden uit oplosbaar antigeen (hoofdstuk 3) of verder waren gezuiverd door passage over sephacryl S-200 kolommen (hoofdstuk 4), werd gevonden dat de specificiteit van de LAI-test kon worden opgevoerd tot ongeveer 95%. Echter, de gevoeligheid van de test bleef gelijk (±70%) terwijl ook de houdbaarheid van de extracten niet verbeterd bleek te zijn.

Omdat de voortdurende schaarste aan geschikt tumormateriaal bij de mens verhinderde dat goede vorderingen konden worden gemaakt met de verdere zuivering van tumor-geassocieerde antigenen, werden parallel-studies opgezet bij ratten. Er werd gekozen voor drie verschillende tumormodellen met toenemende immunogeniciteit: een niet-immunogeen sarcoom (LS175) bij BN ratten, een zwak immunogeen coloncarcinoom (CC531) bij WAG ratten en een sterk immunogeen huidcarcinoom (1618), eveneens bij de WAG rat. In hoofdstuk 5 worden de LAI

resultaten beschreven die werden waargenomen met bovengenoemde, overentbare, tumoren. Het bleek dat tumordragende ratten, ongeacht het gebruikte model, slechts incidenteel en positieve LAI-reactie te zien gaven. Ook indien in plaats van met leukocyten uit het perifere bloed, gewerkt werd met lymfoide cellen uit de peritoneaal-holte bleven de resultaten marginaal.

Om te onderzoeken of de slechte LAI resultaten bij de rat te wijten waren aan een te lage concentratie aan relevant tumor antigeen in de gebruikte tumor-extracten werden vervolg experimenten uitgevoerd in het CC531 model (hoofdstuk 6). Tumorextract werd "gezuiverd" met behulp van Sephacryl S-200 kolommen en de LAI testen werden uitgevoerd met leukocyten uit het bloed of lymfocyten uit de buikholte. De resultaten bleven onbevredigend. Ook een vergelijkend onderzoek met de verschillende fracties uit de zuiveringsprocedure vermocht niet aan te geven dat er tijdens de bewerking van de tumor LAI-reactief materiaal verloren was gegaan.

In hoofdstuk 7 wordt in een eindevaluatie een oordeel gegeven over de betekenis van de LAI-test in de kliniek. Bovendien wordt ingegaan op de mogelijke redenen van het falen van de LAI-test bij de rat.

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