IMMUNOTHERAPY IN MICE OF VIRALLY INDUCED TUMORS USING SYNGENEIC MONOCLONAL ANTIBODIES

Immunotherapie van viraal geïnduceerde tumoren in muizen met syngene monoclonale antilichamen

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CHAPTER I

GENERAL INTRODUCTION

1. History

Cancer immunotherapy may be defined as the use or modification of elements of the immune system to limit or eliminate neoplastic growth. This has been an appealing concept since Ehrlich in 1900 postulated the presence of differences between surface membrane antigens of normal and tumor cells allowing an immunocompetent organism to recognize and destroy abnormal cells.

The existence of tumor restricted immunity was first shown by Gross (1943) who found resistance against tumorogenic doses of a chemically induced sarcoma if mice were sensitized first with a small number of the same tumor cells. These findings were confirmed later by Foley (1953). Using inbred mice, Prehn and Main (1957) and Klein et al. (1960) demonstrated the specific resistance against rechallenge with a previously excised, methylcholanthrene-induced tumor. The tumor was accepted by syngeneic mice not previously exposed to the tumor.

Interest arose which effector mechanism dominated tumor rejection. Mitchison showed in 1954 by transfer experiments the ability of presensitized lymphocytes, obtained from draining lymph nodes of non-susceptible tumor-bearing mice, to accelerate tumor graft rejection in other mice, whereas serum alone failed to do so. Gorer and Amos (1956), however, were able to demonstrate protection against an inoculum of tumor cells by passive immunization with hyperimmune serum.

Further exploration of the immune system led to delineation of the immune system into cellular (T cell) and humoral (B cell) components (Cooper et al., 1966; rev. Roitt et al., 1969). Meanwhile Burnet formulated his famous immunosurveillance theory (Burnet, rev. 1971) based on the postulation that tumor-specific or tumor-associated antigens exist, distinct from the non-transformed cells from which they arose and that an immune response may occur against these antigens. After this period tumor immunology underwent an acceleration especially by the development of the hybridoma technology (Köhler and Milstein, 1975) and by the introduction of new techniques in molecular biology (rev. Weinberg, 1985). New areas of the immune system were revealed and

tumor surveillance appeared not anymore exclusively a T cell restricted phenomenon, but the involvement of humoral antibodies, macrophages and natural killer cells has been documented as well (rev. Wheelock and Robinson, 1983; rev. Den Otter, 1986).

As regards the concept of tumor-specific antigens, which are supposed to play a key role in many therapeutical approaches, much scepticism has raised about their very existence and immunogenicity, except for virally, chemically or physically induced experimental tumors (rev. Weiss, 1980). Therefore, a more accurate term might presently be: tumor-associated antigens. In view of the heterogeneity of these antigens the definition remains also difficult, but may be at best formulated as "a molecule which presence renders the cancer cell quantitatively or qualitatively different from its normal counterpart" (Sulitzeanu, 1985).

The use of immunogenic tumors in animals as a model for human immunotherapy has been the subject of debate (Hewitt, 1982; Herberman, 1983). It seems however reasonable to assume that unsuccessful therapy in such models precludes its clinical use (rev. Ross and Steele, 1984).

The development of tumor immunology has greatly inspired the use of new immunotherapeutical methods. However, the clinical applications reported are far from conclusive (rev. Oldham, 1983; rev. Rosenberg, 1984; rev. Reisfeld and Cheresh, 1985), sporadically showing promising results (Miller et al., 1982; Rosenberg et al., 1985). It is obvious that much more experimental work has to be done before different forms of immunotherapy find their place between the traditional modalities as surgery, radiotherapy and chemotherapy.

2. Aim and outline of the study

This thesis deals with one variant of immunotherapy, namely the use of tumor-specific antibodies to induce tumor destruction. The experimental work falls apart into four succeeding phases: a) definition of the model, which means the choice of the animal and the tumor, the generation and characterization of the monoclonal antibodies; b) testing the monoclonal antibodies for

their ability to find the right target in vivo; c) testing these antibodies on the suitability for immunotherapeutical purposes; d) investigations on the underlying mechanism which may lead to tumor cell destruction.

An artificial tumor metastasis model was chosen to imitate in a controlled way metastatic cancer, which is still the major problem in conventional therapy of malignant neoplasias.

The non-producing Rauscher virus induced myeloid leukemic cell line RMB-1 was selected (De Both et al., 1981). Experiments carried out in the order as outlined above are reported in the Chapters II - VI. Chapter II deals with the characterization of two monoclonal antibodies directed against the viral protein p12, its precursors and a glycosylated polyprotein. In Chapter III results on specific in vivo targeting of both monoclonal antibodies are reported and discussed. Chapter IV deals with the use of one monoclonal antibody for immunotherapy in a disseminated tumor model; its possibilities and limits are investigated. In Chapter V an attempt is made to explore the underlying mechanism by carrying out in vivo and in vitro experiments and by histological studies. Chapter VI is directed to one aspect of the underlying mechanism, namely delayed-type hypersensitivity elicited by the tumor cells in the immunocompetent host. In Chapter VII the model is discussed in the context of the contemporary literature.

3. RNA tumor viruses

3.1 General outline

The RNA tumor viruses (oncovirinae) are a subfamily of the retroviruses (retroviridae). The former were among the first agents that were discovered to cause tumors in animals. Initially the transmission of chicken erythroleukemias and sarcomas by cell free extracts was described by Ellerman and Bang (1908) and by Rous (1910), but it took 40 years to demonstrate this for murine leukemias in 1951 by Gross (rev. Gross, 1970). Since that time many other leukemia inducing murine retroviruses have been described, some of which cause myeloid and erythroid leukemias as the Graffi, Friend and Rauscher virus (rev. Gross, 1970). RNA tumor viruses have also been found in many other vertebrates e.g. birds, other rodents, cats and monkeys (rev. Stephenson et al., 1978; rev. Weiss et al., 1982). During the past years also human retroviruses are isolated, which induce (T cell) leukemias (HTLV I, II) and the acquired immune deficiency syndrome (HIV) (rev. Wong-Staal and Gallo, 1985; rev. Weiss, 1987).

3.2 Murine leukemia viruses

Murine leukemia viruses (MuLV) belong to the C-type RNA tumor viruses and measure about 1000 Å in diameter. They are composed of an envelope covering the centrally located viral core (Fig. 1). The envelope consists of a lipid bilayer from which knobs project consisting of the major envelope glycoprotein gp70, loosely connected to the surface by the transmembrane envelope protein p15E. Between these stalks the protein p12E is present (rev. Bolognesi et al., 1978). The internal viral structure is constituted by four proteins of p15, p12, p30 and p10. The opinions about the composition of the inner coat between envelope and core are controversial. Some authors suggest that p15 is present in the inner coat (Barbacid and Aaronson, 1978; Van der Ven et al., 1978), whereas others assume that p15 is located just exterior to the core (rev. Bolognesi et al., 1978).

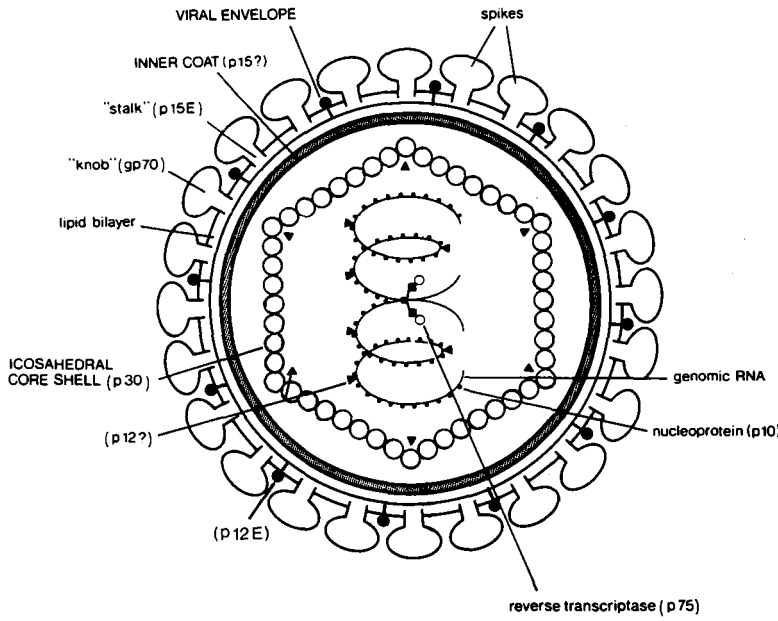

Fig. 1. Type C virus particle (modified to W. Hesselink).

The principal constituent of the core shell is p30. P10 forms a ribonucleoprotein complex with the viral RNA, which consists of two identical 35S molecules linked together by hydrogen bonds. P12, which is a phosphoprotein with the property to bind specifically to the homologous genomic RNA (Sen and Todaro, 1976), is most probably located in the core. The morphological aspects and the composition of type C RNA viruses are reviewed by Montelaro and Bolognesi (1978).

3.3 The viral genome

The RNA genome of retroviruses consists of three regions called gag, pol and env, which encode respectively for viral core proteins, the enzyme reverse transcriptase and the envelope glycoprotein, respectively. On the 5'- and 3'-sites repeats are present called RU5 and RU3. The viral RNA has a structure, which is homologous to that of messenger RNA having a cap site on the 5'-end and a poly-adenosine tail on the 3'-end.

3.4 Viral replication

After adsorption of the virion to cellular receptors the virus is endocytosed and the particle dismantled. The viral RNA comes free in the cytoplasm. By means of reverse transcriptase, a RNA-dependent DNA polymerase, present in the virus particle, a DNA copy is formed, which is complementary to the viral RNA. After degradation of the RNA, the DNA is duplicated and transported to the cell nucleus. The linear DNA is circularized by DNA ligase into two circular forms of which only one is integrated as provirus into the cellular DNA. In this manner genetic information is added to the genome of the infected cell and if such cells divide, transmitted to the daughter cells. In general the integration is at random.

3.5 Provirus

The structure of the proviral genome differs from the viral genome in that at both sites a long terminal repeat is present, called LTR, which consists of U5, R and U3.

The LTR contains enhancer and promoter elements by which viral RNA synthesis can be induced. The unspliced RNA of 35S is incorporated into the virion, whereas spliced RNA is translated into viral proteins.

3.6 Virally encoded protein synthesis

The gag region of the virus codes for a 65 kDa precursor protein, which is cleaved via intermediates into the core proteins p15, p12, p30 and p10 (Fig. 2).
The gag proteins are also expressed on the membranes of virus infected cells as part of a glycosylated polyprotein. The pol region encodes for reverse transcriptase p75, a smaller protein of 14 kDa which is responsible for proteolytic cleavage of the precursor $Pr200^{gag-pol}$ and an enzyme called integrase (p46), which is involved in proviral integration.

The envelope glycoprotein gp70 and the transmembrane protein p15E are formed from a precursor $gPr80^{env}$. The p15E is partly cleaved into p12E. The viral envelope glycoproteins migrate to the cell membrane and are inserted in the cell surface. Subse-

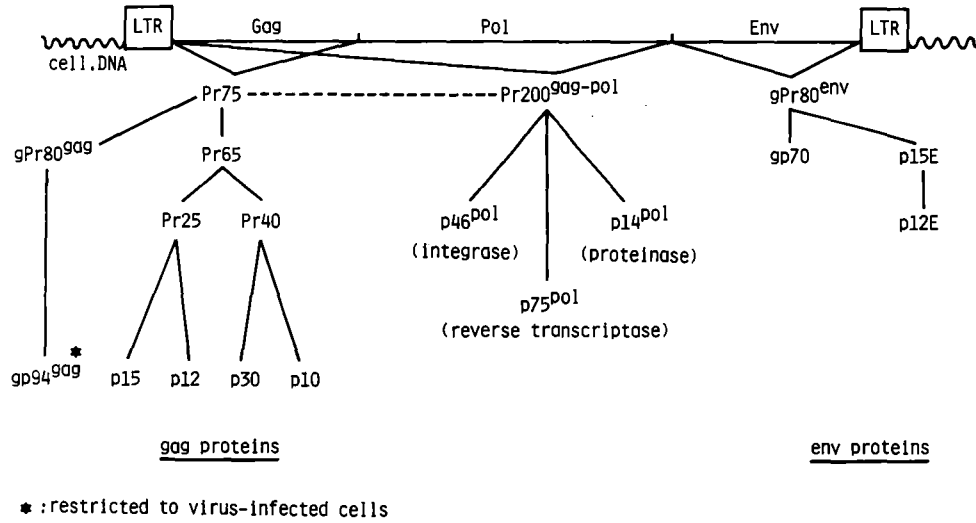

Fig. 2. Rauscher proviral genome and processing of its precursor proteins.

quently, the viral core proteins, reverse transcriptase and the genomic RNA are transported to this site.

After assembly and packaging the viral particle leaves the cell by budding. Since in the same cells multiple integrations can take place and since one provirus can give rise to the synthesis of many virus particles, the virus is strongly amplified in infected animals.

The viral replication and protein processing are reviewed by Goff and Lobel (1987).

3.7 Viral host range

The host range of MuLV is mainly determined by the properties of the viral envelope glycoprotein gp70, which interacts with the cellular receptor (DeLarco and Todaro, 1976; Cloyd et al., 1985).

According to their host range four virus classes are known:
ecotropic : only infectious for murine cells;
xenotropic : only infectious for non-murine cells;
amphotropic: infectious for murine and non-murine cells;

dualtropic : mink cell focus-inducing (MCF): identical in host range to amphotropic viruses, but are distinguished from the latter serologically and by their ability to induce cytopathological changes in mink lung cells (Hartley et al., 1977).

MCF viruses are recombinants derived by substitution of a part of the ecotropic sequences by endogenous xenotropic-like sequences in the viral *env* gene region (Chattopadhyay et al., 1981; rev. Famulari, 1983; Fischinger et al., 1985). Recently another class of recombinant viruses has been reported which are antigenically and structurally similar to MCF viruses, but have an ecotropic host range (Cloyd and Chattopadhyay, 1986). Their pathogenicity is uncertain.

Another classification is made on the basis of susceptibility of mouse strains to various viruses. Two classes are discerned: N- (NIH/Swiss cells) and B- (BALB/c cells) tropic viruses. This tropism is determined by the presence of the *Fv*-$1^{n/n}$ or *Fv*-$1^{b/b}$ genotype (Hartley et al., 1970). *Fv*-$1^{n/b}$ mice are resistent to both N- and B-tropic viruses (rev. Meruelo and Bach, 1983).

3.8 Viral strains

Retrovirus strains which induce leukemias can be divided into *acute* and *chronic* viruses. The former group includes the Friend and Rauscher virus, induces an acute erythroid leukemia and kill the infected mice within 4 weeks. *Chronic* viruses as the Gross, AKR and Moloney leukemia virus act more slowly and are lethal after more than half a year.

The *acute* leukemia viruses consist of a replication defective component and a replication competent virus which functions as a helper virus and complements the defective parts of the former.

The avian defective leukemia viruses contain an oncogene (see further on) and in mice the defective virus has recombined with endogenous xenotropic viral sequences particularly in the region of the *env* gene.

In Rauscher virus the defective component is called Spleen Focus Forming Virus (R-SFFV), whereas the helper virus is denominated as R-MuLV. The helper virus can be purified separately (Mol

et al., 1982) and induces chronic B cell or T cell leukemias (Reddy et al., 1980; de Both et al., 1983) and occasionally myeloid leukemias (de Both et al., 1981, 1985). The murine sarcoma virus, which is often used in immunotherapy studies, is a recombinant of the Moloney leukemia virus with the mos oncogene and induces rhabdomyosarcomas (Papkoff et al., 1982).

The Rauscher, Friend and Moloney viruses show homology both in nucleotide sequences and in serology and are often referred to as the FMR group. THe AKR and Gross viruses are also mutually related (rev. Old and Stockert, 1977).

3.9 Leukemogenesis

The acute leukemia viruses cause a rapidly progressive leukemia by the direct mitogenic or transforming action of the defective viral component, which has undergone recombination with cellular sequences. The proliferation of specific leukemic target cells becomes disregulated by the direct action of the virus. It is assumed, that the virus (or viral proteins) interacts with specific receptors of growth factors or mimic these growth factors. In the case of acute Rauscher virus induced erythroleukemias (Rauscher, 1962), factors involved in erythroid differentiation would interact with retroviral proteins, especially with the env encoded deleted glycoprotein gp 55 (Ruta et al., 1983; Ruscetti and Wolf, 1985). However, the leukemic blasts are still capable of differentiating in long-lived erythrocytes as in the polycythemia inducing variant of Friend virus (FVP) (Tambourin et al., 1973) or in short-lived erythrocytes resulting in anemia as in Rauscher leukemia (de Both et al., 1980).

In chronic leukemias the leukemogenesis is less well understood. Chronic leukemia viruses are sometimes already present in uninfected animals as endogenous viruses which may become activated with age. In other strains newborn mice have to be infected to prevent immunological rejection of the virus and to induce leukemia. Different hypotheses exist how these viruses which do not carry themselves an oncogene or dualtropic viruses induce leukemia.

a. Generation of Mink Cell Focus Forming (MCF) viruses

Some authors assume that ecotropic viruses have to recombine with xenotropic virus sequences to form dualtropic viruses which can also infect non-rodent cells (Hartley et al., 1977; rev. Famulari, 1983; Fischinger et al., 1985). These dualtropic viruses are thus able to bind to different receptors than the original ecotropic virus, resulting in additional infections. MCF viruses can be isolated from many murine lymphomas and leukemias. When mice are infected with this type of virus, leukemias can be induced, but only after a long latent period (van Griensven and Vogt, 1980; Zijlstra et al., 1984). It is not precisely known how these viruses cause leukemia, but it may be that infection with these viruses results in a second hit as part of a multistep mechanism, which leads to leukemogenesis. Otherwise the altered envelope glycoproteins may be involved in the induction or maintainance of the leukemic state (rev. Neil and Forrest, 1987).

b. Insertional mutagenesis

Although proviral integration is at random, it is found in avian and murine lymphomas, that proviruses may activate cellular genes, especially proto-oncogenes which are the cellular equivalents of oncogenes present in many transforming retroviruses. Generally these proto-oncogenes represent loci which products transduce signals involved in the regulation of cell proliferation. At present, more than 20 of such proto-oncogenes are known.

It is assumed that sequences of the viral long terminal repeat (LTR) of ecotropic as well as MCF viruses promote or enhance the expression of proto-oncogenes lying in the vicinity of the integrated provirus (Hayward et al., 1981; Corcoran et al., 1984). A number of important integration sites lying in a particular chromosomal region have been discovered. Since these sites do not represent genes which are present in transforming retroviruses, they are called putative oncogenes.

One of the first detected was *int*-1 in virally induced mammary carcinomas (Nusse and Varmus, 1982). In murine lymphomas induced by AKR and Moloney virus *pim*-1 is described (Cuypers et

al., 1986). Recently also in Friend helper virus induced lymphomas and in myeloid leukemias specific integration sites are detected called fis-1 (Silver and Kozak, 1986) and fim-1 and -2 (Sola et al., 1986). However, in all these cases the integration sites reported are only detected in part of the leukemias. As these reported integration sites do not seem obligatory, activation of other oncogenes may also trigger leukemogenesis.

c. Induction of rearrangements and amplification of oncogenes

In a number of virally induced plasmacytomas a translocation is reported between chromosome 15 and 12. By this process the myc oncogene on chromosome 15 is transferred to a site near to enhancers and promotors involved in immunoglobin synthesis lying on chromosome 12 (Stanton et al., 1983). Often the oncogenic transcripts are truncated.

In the Rauscher virus induced myeloid cell line (RMB-1), which is used for our experiments, a trisomy of chromosome 15 is reported (Hagemeijer et al., 1982), similarly as in lymphomas induced by Moloney leukemia virus (Spira et al., 1979). It is suggested, that these extra chromosomes impose a proliferative advantage to the cells, but it is not known how, and if, these extra chromosomes as such induce leukemogenesis (Klein, 1981).

d. Other hypotheses

Several other, sometimes more indirectly operating mechanisms are postulated.

An interesting hypothesis has been proposed by McGrath et al. (1980). The concept is that T cells are induced to proliferate after binding of leukemia viruses to mitogenic receptors. The prediction that proliferation could be blocked by substances that interfere with virus-receptor interaction could be verified (McGrath et al., 1980). It has been suggested that chronic antigenic stimulation caused by viraemia, leads to continuous production of lymphokines as IL-2 and IL-3 by lymphocytes. This may in turn facilitate the generation of lymphomas (Ihle et al., 1982), possibly by increasing the rate of mutations during extensive proliferation.

Otherwise leukemogenesis may be privileged by immunosuppression. Viral derivatives as the envelope protein p15E may act as

immunosuppressive agents as suggested by in vitro studies (Mathes et al., 1978; rev. Bendinelli, 1985), creating an opportunity for neoplastic growth.

3.10 Genetic influence of the host

The degree of resistance to viraemia and to infected or transformed cells may determine the progress of leukemogenesis. The susceptibility to viraemia as well as the expression of viral proteins, needed for immune recognition, are under genetic influence of the host, sometimes closely associated with the murine major histocompatibility (H-2) complex (rev. Meruelo and Bach, 1983). Otherwise virus infection may be influenced by the Fv-1 locus, which interacts with the integration of viral DNA (Jolicoeur and Baltimore, 1976; Jolicoeur and Rassart, 1981). The immune response to viraemia and cells expressing viral proteins is generally H-2 linked (Vlug et al., 1981, 1983). However it can be associated with non-H-2 genes as exemplified by the Rfv-3 gene, which influences the antibody level in mice infected with the Friend virus complex (Chesebro and Wehrly, 1979).

3.11 Antigenicity of viral proteins

By means of competitive radio-immuno assays (RIA) or virus neutralization studies the viral proteins have been shown to possess antigenic determinants, which can be divided into three groups, present in different proportions on each structural protein.

a) Type specific determinants shared by viruses of the same strain.
b) Group specific determinants shared by viruses of the same species.
c) Interspecies specific determinants shared by viruses of different species.

The distribution of the various determinants is presented in Table I.

Table I

Viral protein	Type	Group	Interspecies	Reference
gp 70	+++	++	(+)	Steeves and Strand, 1974; August et al., 1974; Lilly and Steeves, 1974; Hino et al., 1976; Ihle et al., 1976; Barbacid et al., 1976; Stephenson et al, 1978; Nowinski et al., 1978; Chesebro et al., 1981, 1983; Weiss et al., 1982; Gambke et al., 1984.
p15E		++	++	Schäfer et al., 1975; Ihle et al., 1976; Stephenson et al., 1978; Nowinski et al., 1978; Chesebro et al., 1981, 1983; Gambke et al., 1984.
p12E		++		Nowinski et al., 1978, Gambke et al., 1984.
p30	(+)	+++	++	Lilly and Steeves, 1974; August et al., 1974; Stephenson et al., 1974, 1978; Sherr et al., 1975; Strand and August, 1975; Stephenson et al., 1977; Weiss et al., 1982; Gambke et al., 1984; Chuat et al., 1985.
p15	(+)	++	++	Strand et al., 1974; Barbacid et al., 1976, 1977, 1978; Chesebro et al., 1981, 1983; Weiss et al., 1982.
p12	+++	(+)		Stephenson et al., 1974, 1977, 1978; Lilly and Steeves, 1974; August et al., 1974; Barbacid et al., 1976, 1977; Weiss et al., 1982; Gambke et al., 1984.
p10		++	++	Lilly and Steeves, 1974; August et al., 1974; Barbacid et al., 1976, 1977; Stephenson et al., 1977, 1978; Weiss et al. 1982.
p75		++	+	Parks et al., 1972; Sherr et al., 1975; Panet and Kra-Oz, 1978; Weiss et al., 1982.

3.12 MuLV-induced cell surface antigens

A number of cell surface antigens is expressed on MuLV infected or transformed cells (rev. Bauer, 1974; rev. Old and Stockert, 1977; rev. Kurth et al., 1979). These proteins may be divided into viral envelope proteins, glycosylated gag polyproteins and virus induced cellular proteins. The most well known and some recently reported antigens are presented in Table II.

Table II

Antigen	Tissue distribution	Associated viral protein	References
G1X	Gross MuLV induced leukemic cells, thymocytes of high leukemic (AKR, C58, C3H) and some low leukemia incidence strains (129, A)	ecotropic gp70	Old et al., 1964b; Stockert et al., 1971; Old and Stockert, 1977.
G(rada)	Normal and leukemic lymphoid tissues of high leukemia incidence strains	ecotropic gp70	Obata et al., 1978; Old and Stockert, 1977.
FMR	Friend, Moloney, Rauscher virus infected cells.	ecotropic gp70	Old et al., 1964; Old and Stockert, 1977; Nowinski et al., 1978.
G(B10.A)	B10.AV+ lymphoma cells, RADA1 tumor cells, spleen cells of young adult B10.AV+ and B10.AV- mice	(ecotropic) gp70 (dualtropic)	Vlug et al., 1983.
B6RV2	RadLV induced leukemic cells of C57BL/6 mice	(ecotropic) gp70	Nakayama et al., 1984.
G(erld)	Normal and leukemic lymphoid tissues of high leukemic incidence strains	xenotropic gp70	Old and Stockert, 1977; Obata et al., 1981.
G(aksl2)	AKR leukemic cells and thymocytes from young mice of high leukemia incidence strain	dualtropic gp70	Old and Stockert, 1977; Stockert et al.,1979; Famulari, 1983.
XenCSA	Lymphocytes of all inbred mouse strains (most expressed in high leukemia incidence (AKR, C58, PL) strains)	dualtropic gp70	Morse et al., 1979.
GCSA*	Cells infected with replicating Gross-MuLV	glycosylated gag-polyprotein	Old et al., 1964; Tung et al., 1976; Ledbetter and Nowinski, 1977.
MCSA	Mo-MuLV induced lymphomas	gag proteins env proteins	Karande et al., 1979; Hesseling et al., 1981.
P120	Abelson MuLV transformed cells	gag proteins	Witte et al., 1979.
X1	Hemopoietic tissues of high leukemia incidence strains (AKR, C58, C3H) and BALB/c, A and AKR leukemias	-	Sato et al., 1973; Old and Stockert, 1977.

PC-1	BALB/c myeloma cells and liver, kidney, brain, lymph node and some spleen cells of a number of PC-1+ strains (C3H, A, BALB/c, NZB/BL, AKR, etc.)	-	Old and Stockert, 1977; Takahushi et al., 1979.
TL	Differentiation antigen which may appear on leukemic thymocytes of TL- strains	-	Old and Stockert, 1977.
ML	DBA/2 leukemic cells, mammary tissues and mammary tumors of mice infected with mammary tumor virus.	-	Stück et al., 1974; Old and Stockert, 1977.
PC-2	Plasma cells and plasmacytomas of BALB/c origin	-	Tada et al., 1980.
gp175	Friend, Moloney, Rauscher virus infected cells.	-	Rogers et al., 1984.
YE6/6	Chemically, virally (Mo-MuLV) induced or spontaneous arosen lymphomas and leukemic cells of AKR, BALB/c and DBA/2 origin. induced by AKR, Mo-MuLV or A-MuLV	-	Takei, 1987.

* Identical glycoproteins are described for Friend (Evans et al., 1977), Moloney (Edwards and Fan, 1979) and Rauscher virus infected cells (Schultz et al., 1979).

4. Host anti-tumor response

4.1 The H-2 complex

It is well established that the immune response acts in the context of the major histocompatability complex (MHC), in the mouse called the H-2 complex. The H-2 complex of the mouse is localized on chromosome 17 and can be divided in a K, I, S, D, L, Qa and TLa region. The K, D and L regions encode for the class I MHC products, which control the recognition by the cytotoxic T lymphocytes (CTL), while the I region codes for the class II MHC molecules, which function as restriction elements for helper T cells (rev. Klein and Nagy, 1982; rev. Doherty et al., 1984).

Class I molecules ar found on cells of almost all tissues. The class I genes are highly polymorphic and more than 100 alleles at H-2K and H-2D are known (Doherty et al., 1984). The function of the class I-like TLa and Qa regions is uncertain (rev. Klein and Nagy, 1982).

Class II molecules are expressed mainly by B cells and macrophages and serve as focussing sites for helper T cells. The I region is further divided in an I-A and an I-E domain.

Loci in the S-region encode for some serum proteins as C2, C4 and factor B which are not restrictive for the immune responses. These products are often called Class III molecules.

4.2 Humoral response

It has been shown that against a number of tumors a natural resistance or immunological host response may occur, depending on the properties of the tumor and the host (rev. Wheelock and Robinson, 1983; rev. Meruelo and Bach, 1983; rev. Den Otter, 1986). Both humoral and cellular immune responses may occur, which probably interact in a complicated manner, involving lymphokines and other mediator substances. To study the function of the various parts of the system, often tumors are employed which are highly immunogenic and which are often chemically, physically or virally induced. In these studies the role of antibodies has been explored in immunocompetent hosts,

correlating the antibody level to progression or regression of the tumor (Lamon et al., 1973; rev. Levy and Leclerc, 1977; Key and Haskill, 1981a; Ciavarra and Terres, 1984) and in vitro by analyzing (hyper-)immune sera and measuring their effects on tumor and presumed effector cells (Lamon et al., 1976; Langlois et al., 1981; Kawashima et al., 1985). Also B cell deprived animals were screened for their susceptibility to tumor growth (Gordon et al., 1982). Otherwise the effect of the administration of immune sera on tumor growth and tumor prevention was studied (Gorer and Amos, 1956; Fefer, 1970; Haagenesen et al., 1981; Herlyn and Koprowski, 1982; Lamon et al., 1987). The latter subject will be discussed later.

From all these studies it can be concluded that naturally occurring anti-tumor antibodies may act beneficially, exerting their effect mainly via an antibody dependent cellular cytotoxicity (Shin et al., 1975; Levy et al., 1979; Key and Haskill, 1981b). Sometimes, however, detrimental influences may be observed by the induction of blocking factors which presumably exist of antibody-antigen complexes. These immune complexes can act in an immunosuppressive way, thus enabling tumor growth (Hellström and Hellström, 1969, rev. 1974).

4.3 T cell response

The importance of a T cell mediated response in tumor graft rejection has been acknowledged since three decades (Mitchison, 1954). The function of the several T lymphocyte subsets has been related to separate phenotypes (Cantor and Boyse, 1975a, 1975b; Beverley et al., 1976). Generally Lyt-1+ cells are associated with helper activity. By secretion of lymphokines these cells enhance the generation of CTL and cause activation of macrophages. Lyt-2+3+ lymphocytes on the other hand, are precursors of CTL, and can mediate after activation cytotoxic activity. Also many suppressive activities are mediated by Lyt-2+3+ cells. These classifications are, however, not absolute (rev. Hamaoka and Fujiwara, 1987), partly because the Lyt-1 marker was found to occur on CTL as well. The L3T4 marker was found to correlate more exclusively with the helper T function. (Dialynas et al., 1983).

Mostly, highly immunogenic tumors have been used to study the involvement of T cell mediated rejection (rev. Levy and Leclerc, 1977; rev. North, 1985). The involved T cell subpopulations were analysed by selective elimination of the various subsets with specific monoclonal antibodies and complement. Several approaches have been followed: a) In vitro mainly by testing killer activity of (presensitized) cytotoxic T lymphocytes (Lyt1-2+) using ^{51}Cr or ^{3}H release of previously labelled cells (Lamon et al., 1972; Shiku, 1976); b) In vivo by testing prevention of tumor growth, either by simultaneous injection of tumor and effector cells (Winn-type assay) (Shimizu and Shen, 1979; Prat et al., 1983; Fuyama et al., 1985) or by induction of regression of an established tumor by adoptive transfer of immmune splenocytes (Fernandez-Cruz et al., 1980; Rosenberg et al., 1986). Sometimes athymic, irradiated, bone marrow reconstituted mice were used (Berendt and North, 1980; Dye and North, 1981). Since the T cell growth factor Interleukin 2 (IL-2) has become available (Morgan et al., 1976; Tanaguchi et al., 1983) the use of in vitro expanded immune T cells derived from spleen, draining lymph nodes or tumor infiltrate has been applied for adoptive transfer (rev. Rosenberg et al., 1986; Shu et al., 1987; Chou and Shu, 1987). Another possibility is to augment cytotoxicity and to expand the presensitized effector population by means of a mixed lymphocyte tumor culture (MLTC) (Fernandez-Cruz et al., 1980; Engers et al., 1984; Cheever et al., 1986). By this approach presumably the production of endogenous IL-2 by the T cells is stimulated. In these transfer studies cyclosphosphamide was often used to abrogate suppressor T function and diminish the tumor burden (rev. Rosenberg, 1984; rev. North, 1985; rev. Evans, 1986).

Additionally, regressing and progressing tumors have been analysed histologically (Evans, 1984; Martin et al., 1987). Variable frequencies of T cells were found, probably depending on the model used. A good correlation between the number of the (various) T cells and progression or regression of the tumor was not always evident.

From studies on the phenotype of the effector T lymphocytes in tumor rejection seemingly controversial results have been obtained (rev. Loveland and McKenzie, 1982; rev. North, 1985). In some studies Lyt-1-2+ CTLs were indicated as the major effector cell population (Leclerc and Cantor, 1980a; Engers et al., 1984; Fuyama et al., 1985; Mulé et al., 1987). Others reported the requirement of Lyt-1+2- (L3T4) cells (Fernandez-Cruz et al., 1980; Greenberg et al., 1981, Fujiwara et al., 1984; Bookman et al., 1987). A number of studies underlines the necessity of the presence of Lyt-1+2+ (3+) precursor cells (Shimizu and Shen, 1979; Fuyama et al., 1986; Prat et al., 1983; Leclerc and Cantor, 1980a) or both Lyt-1+2- (L3T4) and Lyt-2+(3+) (Cheever et al., 1986; Chou and Shu, 1987). The Lyt-1+ and Lyt-2+ cells may then continuously be recruited from Lyt-1+2+3+ precursors (Shimizu and Shen, 1979; Leclerc and Cantor, 1980b). Lyt-1+ cells may either function as helper cells for Lyt-2+ CTL (Chou and Shu, 1987; Cheever et al., 1987) or as mediators of a delayed-type hypersensitivity (DTH) response (Bookman et al., 1987). However, the effects of these cell types on tumor cells are uncertain and tumorgrowth seems to be controlled by a network of helper, cytotoxic and suppressor systems with the apparent involvement of complicated idiotypic interactions (rev. Schreiber, 1984; rev. North, 1985; rev. Robins, 1986; rev. Evans, 1986).

4.4 Macrophage anti-tumor response

The presence of macrophages in tumors is a well known phenomenon (rev. Den Otter, 1986). Since macrophages exert many functions, the study of their effect on tumor growth is difficult. Macrophage activation proceeds through different stages via several sequential signals and these different stages are reflected by the expression of various receptors needed for the acquired functions (rev. Adams, 1982; Ralph et al., 1982; Johnson et al., 1983). Macrophages may act in a specific or non-specific way.

In the immune system macrophages play a central role both as antigen presenting cells in the context of class II major histocompatibility gene products and by activating helper T cells by

interleukin 1 (rev. Buus et al., 1987). Macrophages are also reported to act in an immunosuppressive way (Fernandez-Cruz et al., 1982). They can also be rendered cytotoxic by the T cell mediated release of specific macrophage arming factor (Evans et al., 1973; Dullens et al., 1986) or non-specifically by macrophage activating factor (Piessens et al., 1981), which seems to be equivalent to gamma-interferon (Nakajima et al., 1985).

An important finding was that macrophages may play an effector role in antibody dependent cellular cytotoxicity (ADCC), particularly demonstrated in immunotherapeutic models, either acting rapidly or slowly (Shin et al., 1975; Key and Haskill, 1981b; Langlois et al., 1981; Steplewski et al., 1983; Adams et al., 1984). Rapidly acting ADCC is achieved in about 6-8 hours and apparently needs another activation stage than slowly acting ADCC, which has its maximum at 24-72 hours (Johnson et al., 1986).

4.5 Natural killer cell anti-tumor response

According to Woodruff (1987) natural killer (NK) cells may be defined as cells other than macrophages and polymorphonuclear leukocytes derived from non-immunized animals which are cytotoxic for neoplastic targets in the absence of specific antibody. They may be divided into 2 subpopulations on the basis of their surface markers and their lytic activity in vitro, which is for one class more rapid than for the other. Another division was introduced on the basis of genetic, phenotypical and functional differences in a) NK cells, belonging to a different lineage than T, B or myeloid cells; and b) non-MHC restricted CTL (Lanier et al., 1986). At least some NK cells have a similar morphology as large granular lymphocytes being particularly found in spleen, lymph nodes and peripheral blood and in lower frequencies in newborn and old mice (Herberman et al., 1975, 1978; Kiessling et al., 1975).

The nude mouse is known to have considerable NK activity (Minato et al., 1979). The susceptibility to primary tumors and tumor progression after inoculation of tumor cells is generally well-correlated to the absence of NK activity (rev. Woodruff,

1986). The exact working mechanism is unknown. NK cells can be stimulated by interferons (Gidlund et al., 1978). NK cells may have an active role in natural resistance, particularly in the case of virally induced tumors. This is likely due to the stimulatory action of interferon produced by these cells (rev. Den Otter, 1986).

Since in the immunotherapeutical model described in this thesis virally induced tumors are used, the humoral and cellular immune response against leukemia viruses and virus-infected cells will be discussed separately.

5. Immune responses to MuLV and MuLV-associated cellular antigens

5.1 Humoral response to MuLV

Depending on the mouse strain, an antibody response is elicited against exogenous ecotropic and dualtropic (MCF) viruses in newborn or adult mice (rev. Ihle and Lee, 1982; Zijlstra et al., 1985). In general these responses are less effective in newborn mice which can therefore be more successfully infected than adult mice (McCoy et al., 1972). Also against endogenous ecotropic viruses antibody formation takes place, in contrast to endogenous xenotropic viruses to which detectable antibodies are rarely found (rev. Ihle et al., 1982).

The antibody regulating genes have been mapped mainly to the H-2 complex (rev. Meruelo and Bach, 1983), but a regulatory role of non-H-2-linked loci also has been reported (Chesebro and Wehrly, 1979). The antibody response to activated endogenous ecotropic and MCF viruses is predominantly directed to gp70 and p15E, with neutralizing antibodies directed to type specific determinants of gp70 and non-neutralizing antibodies to group and/or interspecies specific determinants of p15E and gp70 (Ihle et al., 1974; Ihle and Lazar, 1977; rev. Ihle et al., 1982; Zijlstra et al., 1985). The antibody response to exogenous ecotropic viruses of the FMR group is also mainly directed to the envelope proteins, but antibodies against p30 and p12 may be present as well (rev. Ihle et al., 1982). A reverse correlation exists between antibody titers and the degree of viraemia, but the presence of neutralizing antibodies in infected and susceptible mice does not always prevent leukemogenesis (Vlug et al., 1980).

5.2 Humoral response to MuLV-infected cells

Apart from an antibody response directed against the virion itself a humoral response may be induced by cell surface antigens present on virally infected or transformed cells (Collins et al., 1978; rev. Kurth et al., 1979; Weinhold et al., 1984). The H-2 dependent generation of some of these antibodies has been established (Sato, 1973; Vlug et al., 1983).

Although a number of cell surface antigens already mentioned previously (3.12) is defined by hetero- or alloimmunization (rev. Old and Stockert, 1977), some of them display a strong immunogenicity in the syngeneic host. Particularly antigens shared by Friend, Moloney and Rauscher virus on infected or transformed cells belong to this group (Old et al., 1964a; rev. Old and Stockert, 1977). In mice with tumors induced by inoculation with the Mo-MuLV/MSV complex, a specific antibody response to these antigens is detectable, particularly in sera of mice with regressing tumors (rev. Levy and Leclerc, 1977). Cytotoxic antibodies to MuLV affected target cells may prevent leukemia by elimination of these cells (rev. Kurth et al., 1979; Vlug et al., 1983).

5.3 T cell response to MuLV-infected or -transformed cells

The recognition of MuLV related antigens in association with class I MHC products by CTL has been well documented (Gomard et al., 1976; Blank and Lilly, 1977; Stukartet al., 1982; rev. Doherty et al., 1984). The CTL response against MuLV-infected cells is determined both by the level of expression of H-2 antigens and of the virus-encoded antigens (Plata et al., 1981; Flyer et al., 1983). Depending on the H-2 haplotype the CTL recognize the viral antigens in association with either K or D region determinants (Blank and Lilly, 1977; Gomard et al., 1980). The level of expression of viral antigens seems also to be affected by H-2 genes (Meruelo and Bach, 1983). The expression of H-2 products themselves on infected cells is highly variable and depends on the mouse strain used. This expression can apparently be influenced by retroviral infection (Meruelo et al., 1978; Flyer et al., 1985). A CTL response to exogenous FMR MuLV can be easily detected in responder strains (Plata and Lilly, 1979), in contrast to the ecotropic AKR MuLV, which is endogenous to most strains of mice. A heterogeneous group of viral determi-nants can be recognized by MuLV specific CTL (Plata, 1982). In Gross and FMR MuLV-infected cells, envelope glycoproteins (Collins et al., 1980; Flyer et al., 1983; Plata et al., 1987) and (glycosylated) gag (poly)proteins are recognized by CTL (Green,

1980; Plata et al., 1983; Van der Hoorn et al., 1986; Flyer et al., 1986; Plata et al., 1987). Initially it was assumed that for distinction between FMR and Gross virus induced tumors the same markers were recognized by CTL (Plata and Lilly, 1979; Plata, 1982) as was found by serological typing (Old et al., 1964b). However, a recent study has suggested that this is only true for tumor-associated antigens, whereas FMR and Gross virus encoded proteins show cross-reactivity at the CTL levels (Flyer et al., 1986). The antigenicity of these virus encoded proteins is apparently determined by class I MHC molecules, but the intensity and specificity of the immune response probably by genes outside the H-2 complex (Plata et al., 1987).

6. Immunotherapy with monoclonal antibodies

6.1 Introduction

Manipulation of the immune system to destruct a tumor is an attractive perspective and has led to investigations on many fields. Gorer and Amos (1956) were among the first to demonstrate protection against a tumor challenge by the use of hyperimmune sera. These studies were followed by many others using syngeneic, allogeneic or heterologous antisera in animals and sometimes in humans (rev. Rosenberg and Terry, 1977). Protection could be demonstrated when antiserum was administered just prior to or following subcutaneous or intraperitoneal inoculation of tumor cells (Gorer and Amos, 1957; Möller, 1964). However, enhancement of tumor growth occurred as well, especially if sera were given prior to tumor injection (Möller, 1964; Attia and Weiss, 1966). Treatment of established tumors was in general without effect except in some studies using sarcomas induced by the Mo-MuLV/MSV complex, which are known to be very immunogenic (Fefer, 1969; Hellström et al., 1969; rev. Levy and Leclerc, 1977). Also in humans a few, not well documented, successes have been claimed (Sumner and Foraker, 1960; Ngu et al., 1967). Successful therapy seemed particularly limited by lack of quantity and specificity of the immune sera. The development of the hybridoma technology raised new hope for the future (Köhler and Milstein, 1975). Since then a number of animal and human studies has been carried out, but with various results (rev. Foon et al., 1982). From these studies a number of factors became clear concerning the use of antibodies for therapy of cancer, which will be mentioned in the following paragraphs.

6.2 Tumor-specific antigens

Tumor specificity is dependent on the presence of antigens uniquely expressed on the tumor, to which antibodies or CTL can be directed. The search for specificity has been a disappointing one and has led to the conviction that save for a few exceptions as idiotype expression on B and T cell lymphomas (rev. Stevenson and Glennie, 1985), the majority of presumed tumor specific

antigenic determinants is expressed only in different frequencies on tumor and normal tissue. Besides that, it was realized that most tumors, although originally monoclonal, soon become heterogenous in many aspects as the result of mutation and selection of (faster growing) tumor cells (rev. Heppner, 1984). The problem of non-specificity is mainly managed by the use of xenografts on nude mice (Herlyn et al., 1980; Shouval et al., 1982; Mujoo et al., 1987) or by the use of virally, chemically or physically induced tumors, which have antigens not expressed on normal host tissues (Kennel et al., 1983; North and Dean, 1983; Key et al., 1983). These artificially induced tumors may often consistently express tumor-associated antigens.

Antigen loss in the cell population may be caused by selection of tumor cells, which do not express this antigen (Young and Hakomori 1981), but also by antigenic modulation as first described for the TL antigen on thymocytes (rev. Old, 1981). During modulation antigen-antibody complexes are redistributed and shed from the cell surface or internalized (rev. Chatenoud and Bach, 1984). This phenomenon is specific and reexpression occurs after withdrawal of the implicated antibody. Antigenic modulation sometimes is the result of bivalent antibody binding as is described for idiotypic antigens on lymphomas (Elliott et al., 1987).

Antigenic modulation has implications for antibody therapy, which means that in these cases either an initial destruction of all tumor cells should be achieved or antibodies to different antigens should be used consecutively (rev. Chatenoud and Bach, 1984). Otherwise the application of univalent antibodies could circumvent the problem of antigenic modulation. Univalent antibodies can be obtained for instance by fusing immune spleen cells with myeloma cell lines which secrete non-compatible light chains (Cobbold and Waldmann, 1984).

6.3 Type and density of antigens

Depending on the target antigen, the antibody-antigen interaction may be inert or affect tumor growth. The latter may be due to growth inhibition and/or cytotoxicity. Antibodies directed to

the transferrin receptor (Sauvage et al., 1987), the epidermal growth factor receptor (Masui et al., 1984), as well as idiotypic determinants (Schreiber and Leibson, 1978) and sometimes not further identified antigens (Vollmers et al., 1985) have been shown to inhibit tumor growth in vitro by specific antigen-antibody interaction.

Antigen density on tumor cells seems equally important for tumor destruction. When an ADCC mechanism is involved the magnitude of effector cell action is influenced (Herlyn et al., 1985). According to the authors, the affinity of antibodies to their epitope appears to be less important.

The obvious consequence for antibody therapy is selection of antibodies directed to antigens which are present in high densities. Alternatively a mixture of several monoclonal antibodies directed to different targets can be used. This approach has been shown to result in a better response (Herlyn et al., 1985; Ceriani et al., 1987) by the cooperation of different monoclonal antibodies mediating ADCC (Ralph and Nakoinz, 1984). This cooperation for ADCC may be the result of an increased amount of antibody due to binding at various epitopes. A higher binding affinity of some mixtures of monoclonal antibodies compared to the affinity of either individual antibody alone, however, has been demonstrated (Ehrlich et al., 1982). This mechanism also may lead to more antibodies bound to the cell surface and may thus contribute to a more effective ADCC.

6.4 Accessibility of antibodies

The accessibility of antibodies to a tumor is important. A number of studies has shown specific accumulation of antibodies labelled with radio-isotopes in the tumor (rev. Goldenberg and Deland, 1982; rev. Begent, 1985; rev. Ford and Casson, 1986).

It has been shown that vascularisation is, as expected, an important issue (Sands et al, 1986). This is particularly demonstrated by autoradiographic studies of subcutaneous tumors where antibody was preferentially found at the, best vascularized, tumor-host interface (Moshakis et al., 1981; Epenetos et al., 1982). It can therefore be expected that small tumor foci in

well-vascularized organs are best accessible to administered antibodies (see Chapter III).

6.5 Species and isotype of the antibody

It has been established that the antibody class and subclass may affect the final outcome of tumor therapy with monoclonal antibodies. Particularly IgG2a monoclonal antibodies have been shown to be effective (Herlyn and Koprowski, 1982; Adams et al., 1984; Denkers et al., 1985; Berinstein and Levy, 1987), similarly as earlier reported for polyclonal IgG2a antibodies (Matthews et al., 1981). Presumably ADCC is the major effector mechanism and although all IgG antibody classes have the ability to mediate ADCC (Ralph and Nakoinz, 1983), IgG2a antibodies have been proven to be particularly effective in this process (Langlois et al., 1981; Kipps et al., 1985; Johnson et al., 1986). IgG2a and IgM are known to fix complement (C1) and may mediate CDC effectively in mice (Roitt et al., 1985).

The species of the antibody is important with respect to sensitization as is the case for xenogeneic or allogeneic antibodies (Villemain et al., 1986; Benjamin et al., 1986). Anti-idiotypic antibodies may arise, which could abrogate the therapeutic effect by blocking the tumor directed antibody or provoke a hypersensitivity reaction (rev. Chatenoud, 1986).

6.6 Quantity of antibodies and frequency of administration

The quantity of antibodies needed for tumor therapy has not been assessed extensively in most studies and is probably dependent on a number of factors as for instance antibody (sub)class, tumor load, accessibility, antigen density and circulating antigen. In most studies therapy is started just after tumor inoculation (Bernstein et al., 1980; Kirch and Hammerling, 1981; Sauvage et al., 1987). In contrast only few authors studied the effect of therapy on an established tumor burden (Badger et al., 1986). Unpurified ascites preparations or purified antibodies were used, the content of a single dose ranging from $\pm$ 1 µg to $\pm$ 6 mg antibody (Scheinberg and Strand, 1982; Badger and Bernstein, 1983). Detrimental effects of very

high doses were seen (Badger and Bernstein, 1983) as previously shown in models using polyclonal antibodies (Lanier et al., 1979). With respect to the frequency of administration of antibody, some authors report one single injection being equal to a multiple dose regimen (Kirch and Hammerling, 1981; Badger and Bernstein, 1983; see Chapter IV), whereas others claim the necessity of multiple injections (Sauvage et al., 1987). Some studies included data on antibody clearance in their approach to define a dose regimen (Scheinberg and Strand, 1982; Denkers et al., 1985). Apparently each model has its own specific requirements and general rules for dose regimens are not available yet.

6.7 Effector mechanisms

As mentioned already, the mechanism by which antibodies affect tumor destruction is generally thought to be based mainly on ADCC. It is presumed that ADCC is mediated particularly by macrophages (Shin et al., 1975; Johnson et al., 1979). This presumption is mainly based on analyses of tumor infiltrates in which they were present in high numbers (Key and Haskill, 1981b; Adams et al., 1984), whereas their ability to mediate ADCC was shown by *in vitro* studies (Nathan et al., 1980; Kawase et al., 1985; Johnson et al., 1986). *In vivo* studies of appropriate (nude) mouse models confirmed this view (Herlyn and Koprowski, 1982; Steplewski et al., 1983). The effectiveness of antibody therapy in C5 deficient mice (Lanier et al., 1980; Badger and Bernstein, 1983) or artificially complement depleted mice (Kennel et al., 1985) made an important role for CDC less likely. A contributive role of CDC, however, is not excluded in some models (rev. Lamon, 1974; Capone et al., 1983).

Based on the present information available, successful immunotherapy with unmodified monoclonal antibodies may be only expected in a non-immunocompromised host. In this context not only the number of effector cells is important but also their functional activity, which may be abrogated in some cases due to for instance antigen-antibody complexes (rev. Hellström and Hellström, 1974). Shortage of effector cells may not be restricted to conditions of systemic suppression, but may also be a

local obstacle to successful immunotherapy as has been described for large tumors (Johnson et al., 1979).

6.8 Future aspects of monoclonal antibody therapy

Although monoclonal antibody therapy of malignant disease seems promising in a number of studies (rev. Foon et al., 1982), further developments are required to improve the final outcome. Several approaches are currently followed:

The use of:

Subsequent injections of monoclonal antibodies to different target antigens (rev. Chatenoud and Bach, 1984) or univalent antibodies (Cobbold and Waldmann, 1984) to prevent modulation of tumor associated antigens.

A mixture of several monoclonal antibodies with different epitope specificities to prevent immunoselection and to enhance ADCC (Ceriani et al., 1987).

Syngeneic antibodies (this thesis) to avoid an anti-immunoglobulin reaction. The preparation of syngeneic human monoclonal antibodies from lymphocytes of patients with cancer and their subsequent use for treatment seems to yield generally few tumor specific responses (rev. Beverley and Riethmuller, 1987).

Chimeric antibodies. Constructs of antibodies in which only the idiotype regions are of xenogeneic origin (rev. Beverley and Riethmuller, 1987). This approach should prevent anti-antibody response, while retaining high specificity by the xenogeneic immunization procedure (rev. Chatenoud, 1986).

Hybrid antibodies. Constructs of antibodies with one site for tumor specific binding and one site directed to the CTL antigen receptor to enhance cell mediated cytotoxicity (Staerz et al., 1985; Perez et al., 1987). The injection of IL-2 activated mononuclear killer cells "armed" (by incubation) with specific monoclonal antibodies is a variation on this ADCC theme (Honsik et al., 1987).

Immunotoxins and -chemotherapeutics. Antibodies are coupled to toxins or chemotherapeutics. The aim is targeting of these toxins (ricin, abrin, diphteriatoxin, etc.) or chemotherapeu-

tical agents (chlorambucil, methotrexate, etc.) to the tumor site in order to obtain specific tumor destruction (rev. Pastan et al., 1986; rev. Ford and Casson, 1986).

Immunoradiotherapy. Specific tumor destruction is here caused by α, β and γ-radiation from isotopes coupled to monoclonal antibodies (rev. Order, 1981,; rev. Ford and Casson, 1986). A similar approach can equally be used for tumor imaging (chapter III; rev. Begent, 1985).

Immunomodulation. Tumor destruction can be induced by activating the appropriate effector cell subset. Immunotherapy by means of stimulation of Lyt-1+2- cells, using monoclonal antibodies directed to these cells has been described (Hollander, 1984). Otherwise antibodies can be used to eliminate undesirable (suppressor) T cell subsets (Cobbold et al., 1984). Another approach is to manipulate idiotype network systems by anti-idiotype monoclonal antibodies activating silent lymphocyte clones, which may elicit a DTH reaction against the tumor (Forstrom et al., 1984; Nelson et al., 1987; rev. Lee et al., 1986) or utilize anti-idiotype antibodies as a vaccine against the tumor (Raychaudhuri et al., 1986; rev. Lee et al., 1986).

Combination therapy. Improvement of monoclonal antibody therapy may be obtained by simultaneous injection with immune modulating lymphokines as IL-2 (Berinstein and Levy, 1987), alpha-interferon (Basham et al., 1987), or by combination with chemotherapeutical agents (Weill-Hillman et al., 1987).

It is clear that this list is incomplete and even more sophisticated approaches will be developed in the future.

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CHAPTER II

SYNGENEIC MONOCLONAL ANTIBODIES DIRECTED AGAINST RAUSCHER VIRUS INDUCED MYELOID LEUKEMIC CELLS: ISOLATION AND CHARACTERIZATION

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SUMMARY

Hybridomas producing syngeneic monoclonal antibodies (MAbs) were prepared by fusion of spleen cells of BALB/c mice, which were immunized with sublethal doses of RMB-1 cells. This cell line originates from a Rauscher virus (R-MuLV)-induced myeloid leukemia and forms tumors when re-inoculated into mice. MAbs were characterized as regards their reactivity against virally and non-virally induced cell lines. Two selected MAbs IC5F5 and 4D2B4 were analyzed further. Their binding to subcellular structures was determined, and so were the properties of the antigens to which they are directed. MAb IC5F5 is of the IgG2a and 4D2B4 of the IgG2b subclass. Both bind to R-MuLV-infected or -transformed cell lines and are not mutually competitive. The antibodies do not react with other murine and human myeloid leukemic cells. As shown by immuno-electron microscopy, these MAbs have affinity to the cell membrane of non-virus producing RMB-1 cells. When lysates of purified virus were analyzed, the MAbs were found to be directed to the gag precursor protein Pr65, and one of them (IC5F5) also to be directed to the core protein p12. In RMB-1 cells, binding occurs to a 50 kDa glycoprotein and 2 proteins of 26 and 29 kDa. Since RMB-1 cells do not produce virus, but express aberrant viral proteins, these MAbs are tumor-specific and useful for immunotherapy.

INTRODUCTION

There has been considerable speculation on the nature and even the existence of tumor-specific antigens since their introduction into oncology. A number of these tumor-associated antigens are virtually absent from the normal tissue of the adult organism, but are present in fetal tissues. Therefore it is assumed that tumor cells carrying these antigens are clonally expanded after oncogenic transformation (Greaves and Yanossy, 1978; Ibsen and Fischman, 1979). This is not true for cells transformed by many tumor viruses. In this type of cells, viral and transforming proteins are synthesized by newly inserted genes (Bishop, 1987). For instance, in cells infected by Rous sarcoma, virus transformation may result in the induction of antibody formation against both viral and transforming proteins (Erickson, 1980). On the other hand, with chronic retroviruses, which do not carry oncogenes, antibody formation is predominantly directed against viral envelope glycoproteins. This antibody formation is largely dependent on the genetic constitution of the infected mice (Ihle et al., 1982). However, the induction of specific virus-associated antigens has also been reported in Friend, Moloney and Rauscher leukemias (Old et al., 1964; Chesebro et al., 1981; Rogers et al., 1984). Most likely these antigens are abnormally processed glycosylated viral core proteins present in infected cells but not in the virion itself (Weiss et al., 1982).

In general, antibody formation against viral and virus-associated antigens is insufficient to prevent acute leukemias as is the case for Rauscher virus-induced erythroid leukemia (Rauscher, 1962), but an immune reaction prevents chronic leukemias when adult mice are injected with helper virus (McCoy et al., 1972). In contrast, when mice are infected neonatally, lymphatic or myeloid leukemias are induced, but after a much longer latency and with a much lower frequency. From one of these leukemias a transplantable myeloid leukemic cell line (RMB-1) was established, which has been adapted to grow permanently in vitro (de Both et al., 1981, 1985).

When adult mice were repeatedly injected with sublethal doses of RMB-1 cells, an immune response against these cells was detected in their sera. We decided therefore to prepare syngeneic MAbs for immunotherapeutic purposes. In this report we describe the properties of these antibodies and of the corresponding antigens.

MATERIAL AND METHODS

Animals

Six-week-old male and female BALB/c mice were obtained from TNO, Zeist, The Netherlands. Primary erythroid leukemias were induced by injecting of cell-free extracts from leukemic spleen tissue in a 1:4 volume (w/v) in phosphate-buffered saline (PBS). Tumors were obtained after subcutaneous or intravenous injection with 10^7 myeloid leukemic RMB-1 cells.

Viruses

Viruses were purified according to the method of Duesberg and Robinson (1966) from culture fluids of the Rauscher virus producing cell lines 3T3CL9 (Mol et al., 1982) and JLSV5 (Wright and Lasfargues, 1965), and from the Friend-virus-producing cell line 643/22N provided by Dr. I.B. Pragnell, Beatson Institute, Glasgow, Scotland and from Moloney-virus-infected NIH 3T3 cells provided by Dr. P. Bentvelzen, TNO, Rijswijk, The Netherlands. AKR virus was a gift from Dr. A. Berns, Netherlands Cancer Institute, Amsterdam.

Cell lines

For Rauscher-virus-transformed cell lines, the erythroid (RED-1), the lymphatic (RLD-1) and the myeloid (RMB-1) cell lines were used (de Both et al., 1978, 1981, 1983). As non-virally induced myeloid leukemic cells the murine cell line WEHI-3B was used (Metcalf et al., 1969). Cell suspensions of thymocytes of 6-week-old mice and erythroblasts from spleens of mice recovering from anemia induced by 2 consecutive daily injections of 0.2 ml of a 1.25% solution of phenylhydrazine in PBS were used as controls. Macrophages were obtained by an intraperitoneal injection of 5 ml Hanks balanced salt solution (HBSS) and aspiration of the fluid. Before use all cells were washed 3 times in HBSS. As

non-virus-producing fibroblastic cells we used 3T3 cells. All cell lines were cultured either in RPMI 1640 or in Dulbecco's minimum essential medium (Gibco) supplemented with 10% fetal calf serum and 100 IU/ml penicillin and 10 µg/ml streptomycin.

Antisera

Goat antisera against Rauscher viral proteins were: anti-gp70, -p30, -p15, -p12 and -p10, which were a gift from Dr. Varrato, Biological Carcinogenesis Branch, NIH, Bethesda, MD. Rabbit-anti-p15E was a gift from Dr. W. van der Ven, University of Nijmegen, The Netherlands.

MAbs and ascites

In general, the procedure for the production of MAbs as described by Köhler and Milstein (1975) was followed. Adult BALB/c mice were inoculated s.c. with sublethal doses of RMB-1 cells once every 4 weeks for a 3 month period. After a final i.v. booster, sera were screened in an enzyme-linked immunosorbent assay (ELISA, see below) for antibody production against RMB-1 cells. If positive, the spleen cells were fused with the P3-X 63-Ag8.653 BALB/c myeloma cell line.

The hybridomas were screened for antibody production by testing the supernatants in an ELISA. Selected hybridomas were subcloned by endpoint dilution and brought in ascites by injecting 15 x 10^6 hybridoma cells intraperitoneally in BALB/c mice, primed with 0.5 ml Pristane (Janssen, Beerse, Belgium) 10-21 days before.

Micro ELISA system

Screening of antibodies was carried out by a semi-quantitative ELISA on Terasaki microtiter well plates using β-galactosidase linked sheep anti-mouse IgG (Amersham, Houten, The Netherlands) as described by Van Soest et al. (1984). As a reference for fluorescence 0.1 µM/ml methyl-umbelliferon was used. As positive control for RMB-1 cells anti-T200 was used.

Immunoperoxidase staining

Paraffin-embedded or frozen tissues of healthy control mice, and of leukemic and tumor bearing mice, were stained in a 2-step reaction, using antibody-containing supernatant and horse-radish peroxidase conjugated to rabbit anti-mouse IgG (dilution 1:100).

As substrate, diaminobenzidine (500 µg/ml) +3% H_2O_2 in PBS was added. Tissues were counterstained with hematoxylin.

Determination of immunoglobulin classes and subclasses

In an Ouchterlony double immunodiffusion assay the immunoglobulin (sub)class of the MAbs was determined by diffusion in agar of the antibodies against rabbit anti-IgA, -IgM, -IgG, -IgG1, -IgG2a, -IgG2b and IgG3 (Nordic, Tilburg, The Netherlands) and subsequently stained with Coomassie Brilliant Blue (1%) in PBS.

Purification and iodinization of MAbs

MAbs were purified by gel filtration on a DEAE-Sephacel column (Pharmacia, Uppsala, Sweden) and by protein A-Sepharose affinity chromatography (Pharmacia) according to Ey et al. (1978).

The method of Bolton and Hunter (1973) was used to prepare 100 µg ^{125}I-labelled MAbs. The antibody was freed from unbound ^{125}I by gel filtration on a Sephadex G50 column in PBS and stored at 4°C. The iodinized MAbs had a maximal specific activity of 4 mCi/nMol and proved to be biologically active in a solid-phase radioimmuno assay and were electrophoretically pure.

Antigen determination

Virus lysates, lysates of whole RMB-1 and WEHI-3B cells, were made in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Nonidet (NP-40), 1 mM phenylmethylsulphonyl-fluoride (PMSF) + 0.01% leupeptin. After treatment with sodium dodecylsulphate (SDS) electrophoresis was carried out using 12.5% polyacrylamide gels. The gels were blotted on nitrocellulose filter (Millipore) according to Burnette (1981). The filters were incubated with either mono- or polyclonal antisera using ^{125}I-labelled protein-A (Amersham) as second step, or directly incubated with ^{125}I-labelled MAbs. After repeated washes the antigen-antibody complexes were visualized by autoradiography. The presence of proteins on the filters was verified by staining with amido black.

To verify whether the antigen was glycosylated or not, cell lysates were loaded on an antibody column prepared by coupling the antibody to CNBr Sepharose (Pharmacia). Antigen was eluted from the column with 3M KSCN in PBS, the eluate was dialyzed overnight at 4°C against 500 volumes of PBS, concentrated with

Amicon Centricon-10 tubes and analyzed in Western blots. Glycosylation of proteins could be shown by staining according to Clegg (1982).

Competition radioimmuno assay

Microtiter well trays coated with RMB-1 were incubated with different dilutions of IC5F5 and 4D2B4 ascites in PBS + 2% BSA for 1 hr at 37°C. After several washes with PBS + 0.02% gelatin trays of both series were incubated with saturating dilutions of ^{125}I-labelled IC5F5 or 4D2B4 in PBS + 2% BSA for 1 hr at 37°C. After thoroughly washing, the wells were cut out and counted in a LKB gamma counter. Results are expressed as percentage of amount of radioactivity bound by non-preincubated cells. The competition was considered positive when a decrease of radioactivity less than 50% of the maximal binding was measured with increasing concentration of unlabelled MAbs.

Antigen density

Antigen density and affinity was determined according to Scatchard (1949). Briefly, RMB-1 cells were incubated in a total volume of 100 µl 20 mM HEPES in HBSS plus 0.125% gelatin containing various amounts of ^{125}I-IC5F5 (0.01-10 nM) for 90 min at room temperature. For the determination of non-specifically bound ^{125}I-IC5F5, cells were incubated with various amounts of ^{125}I-IC5F5 in the presence of (1×10^{6} M) unlabelled IC5F5. After incubation, the cells were washed with ice-cold HBSS plus HEPES to remove unbound IC5F5. Radioactivity bound to the cells was determined in an LKB-1280 ultragamma counter.

Immuno-electron microscopy

Two million RMB-1 and RLD-1 cells were suspended and fixed in 1.5% v/v paraformaldehyde + 0.1% v/v glutaraldehyde buffered in 0.1 M sodium phosphate buffer, pH 7.2, at 0-4°C for 1 hr. After washing, the pellet was resuspended in 2% agar (Difco, Detroit, MI) and treated further according to Daems and Koerten (1978). The agar pellet was minced into 1 mm cubes and embedded in Lowicryl K4M (Balzers, Liechtenstein) at -40°C. Ultra-thin Lowicryl sections were collected on carbon-coated Formvar-filmed mesh 100 copper grids. Virus was directly dried on the same type of grids. Visualization of the MAbs was realized with 10 nM

colloidal gold coated with goat anti-mouse antiserum (Janssen). As control, phosphate buffer was used instead of MAb, Sections were stained with uranyl acetate and lead citrate. Transmission micrographs were made on a Philips EM 300 electron microscope at 80 kV.

RESULTS

Production and selection of MAbs

In the sera of mice immunized with sublethal doses of RMB-1 cells a syngeneic immune reaction against tumor cells can be detected. Six out of 600 hybridomas made from the spleens of these mice were selected. These hybridomas produced MAbs against Rauscher-induced myeloid leukemic (RMB-1) cells, but not against control erythroblasts, thymocytes and macrophages. Two MAbs, IC5 and 4D2, were subcloned and 2 subclones IC5F5 and 4D2B4 were selected. The results shown in Figure 1 demonstrate that the binding is restricted to the Rauscher virus-transformed or Rauscher virus-producing cell lines with the highest affinity for the RMB-1 cell line against which the antibodies were prepared. Both antibodies had identical reaction patterns, IC5F5 displaying the most pronounced activity. In an Ouchterlony double diffusion assay IC5F5 was shown to be of the IgG2a and 4D2B4 of the IgG2b subclass.

Immunoperoxidase staining

Since one of the reasons for generating the MAbs was the need for application in an immunotherapy model, we investigated their affinity to tumor versus control tissues and the intracellular antigen distribution. The immunoperoxidase assay was carried out on liquid-nitrogen-frozen or paraffin-embedded tissue of fetal and adult control mice or RMB-1 tumor bearing adult mice. In all cases for IC5F5 (Fig. 2) a strong and for 4D2B4 a weaker reactivity was observed for tumor cells, whereas control tissue remained negative.

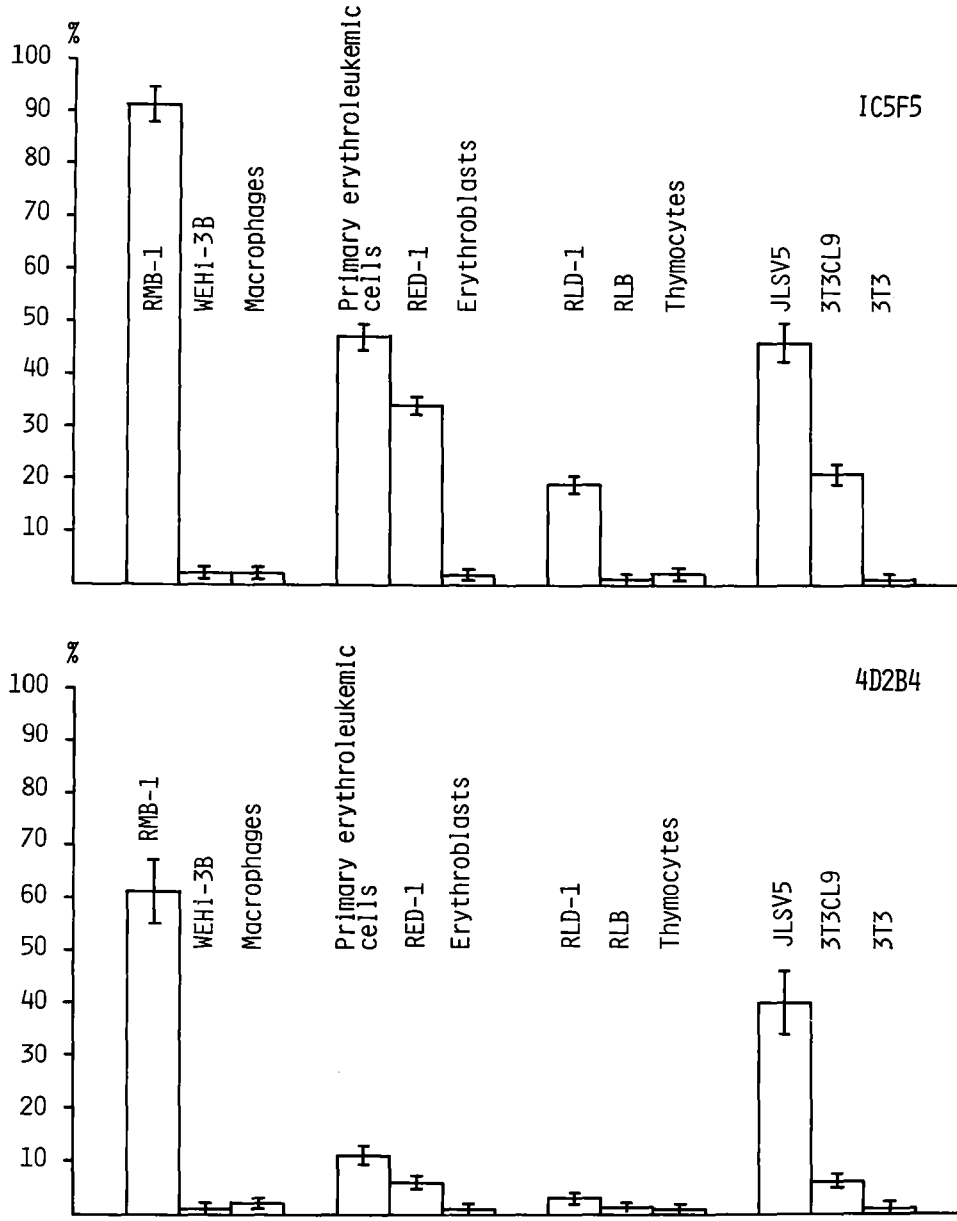

Fig. 1. Reactivity of monoclonal antibodies IC5F5 (a) and 4D2B4 (b) with R-MuLV infected and uninfected cell lines. Data from ELISAs are expressed as percentage of fluorescence of standard (100%) of 0.1 μM/ml Methylumbelliferon. RED-1, Rauscher virus induced erythroid leukemic cells; RMB-1, myeloid leukemic cells; RLD-1, RLB lymphatic leukemic cells. JLSV5, Rauscher virus producing cell line; 3T3-CL9, Rauscher helper-virus-producing cell line; WEHI, non-virally induced myeloid leukemic cells.

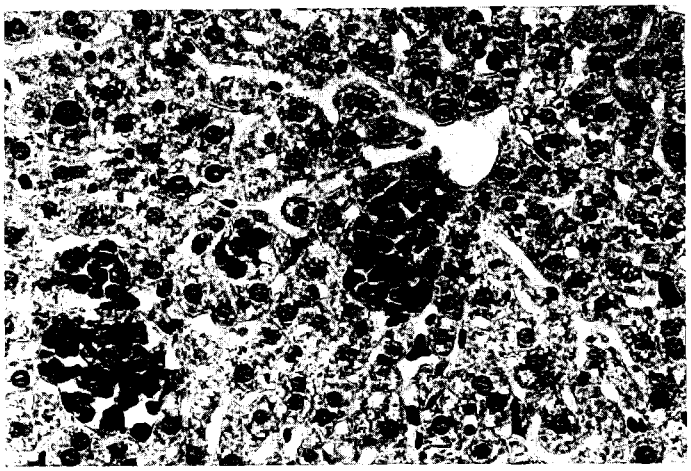

Fig. 2. Immunoperoxidase staining of IC5F5 in liver section of a mouse injected 3 days previously with myeloid leukemic cells

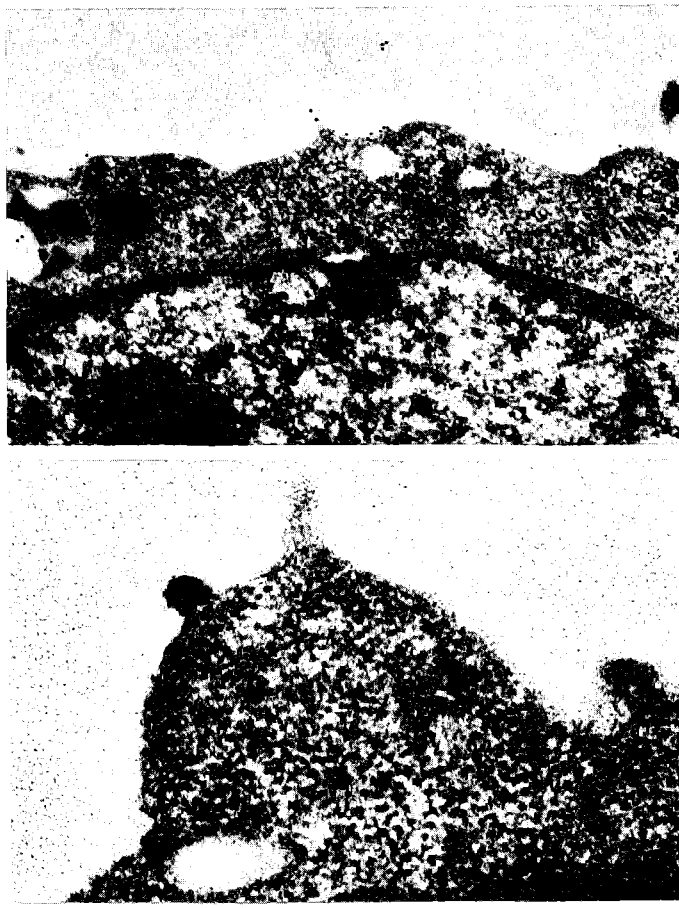

Fig. 3. Immuno-electron microscopy of cells incubated with IC5F5 and gold labelled goat anti-mouse IgG: (a) non virus producing RMB-1 cells; (b) virus producing RLD-1 cells.

Immuno-electron microscopy

The membrane-bound localization of IC5F5 in RMB-1 cells was demonstrated in more detail by immuno-electron microscopy. As seen in Figure 3 many gold particles were present along the cell membrane of this non-producer cell line. Immuno-electron microscopy of the virus-producing, transformed cell line RLD-1 demonstrated concentration of gold label around budding and free virus particles. With 4D2B4 a similar distribution was found.

Antigen determination

a. Rauscher virus. Since the antigen is virus-associated, as shown by the foregoing results, ^{125}I-labelled antibodies were tested against R-MuLV lysates using the Western blot technique. Simultaneously these lysates were tested with polyclonal antisera directed against the known viral proteins p10, p12, p15, p30, gp70 and p15E.

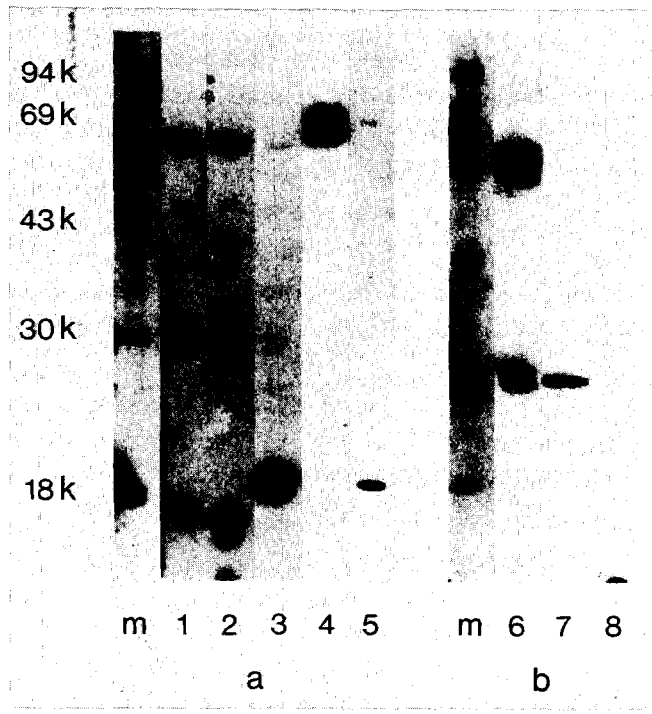

Fig. 4. Screening of R-MuLV lysates with IC5F5 (a) and 4D2B4 (b) and different antiviral antisera. (a) M = markers; lane 1, IC5F5; lane 2, anti-p12; lane 3, anti-p15; lane 4, anti-gp70; lane 5, anti-p15E. (b) M = markers; lane 6, 4D2B4; lane 7, anti-p30; lane 8, anti-p10.

As shown in Figure 4, IC5F5 shows a binding activity identical to that of the p12 sera, intensively staining the gag precursor $Pr65^{gag}$, a doublet at 29 kDa (probably identical to the precursor $Pr27^{gag}$) and a low-molecular weight protein. The pattern is identical to that of anti-p12. The relatively large amount of viral precursor proteins was in accordance with 50-70% immature particles in our virus preparations (electron microscopic data not shown). In addition to staining of p15, the precursor proteins $Pr65^{gag}$ and $Pr27^{gag}$ were stained weakly by anti-p15 sera. With 4D2B4 only, affinity was found for the precursor $Pr27^{gag}$ and $Pr65^{gag}$ proteins but not for p12.

To determine the virus specificity lysates of R-MuLV, F-MuLV, Mo-MuLV and AKR virus were tested by Western blotting against IC5F5 and 4D2B4. R-MuLV and F-MuLV were recognized by both MAbs.

b. RMB-1 cells. Since the antibodies were generated against the RMB-1 cell line, lysates of RMB-1 were compared with lysates of the non-virally-induced WEHI-3B myeloid cell line as control. As reference, lysates of R-MuLV were tested simultaneously. As shown in Figure 5, a cluster of bands was detected in RMB-1 with ^{125}I-labelled IC5F5 showing molecular weights between 48 and 50 kDa. Besides these proteins, 2 circumscript bands were recognized at 29 and 25 kDa. The 29 kDa band is probably also identical to the $Pr27^{gag}$. The low-molecular-weight p12 protein was never detected in cell lysates. ^{125}I-4D2B4 showed only a very weak band of 50 kDa which was hardly visible even after a long exposure time. The WEHI-3B cell lysate did not stain at all with either IC5F5 or 4D2B4.

Staining of the glycosylated residues on the Western blot filters as described in "Material and Methods", showed a positive reaction with the 50 kDa protein in RMB-1 lysates, eluted from an affinity column to which the MAbs IC5F5 was bound, whereas the 29 and 26 kDa proteins did not stain (Fig. 6). These results indicate that at least some of the proteins recognized on RMB-1 by IC5F5 do not belong to the known (precursor) viral proteins and suggest a membrane-bound glycosylated polyprotein of 50 kDa expressing p12 determinants.

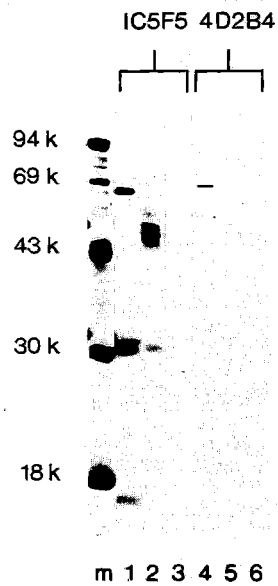

Fig. 5. Screening of lysates of R-MuLV (lanes 1,4), induced RMB-1 cells (lanes 2,5), and non-virus induced myeloid leukemic (WEHI) cells (lanes 3,6) with ^{125}I-labelled IC5F5 and 4D2B4.

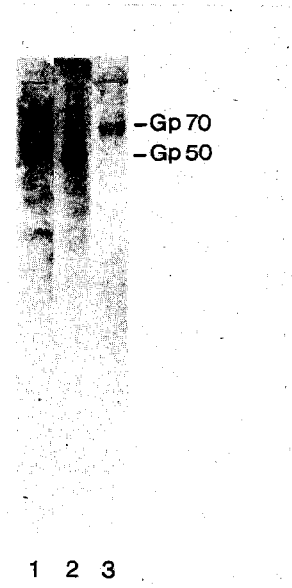

Fig. 6. Glycoprotein staining of antigens eluted from a Sepharose CNBr column to which IC5F5 was bound. Lane 1, total lysates of RMB-1 cells; lane 2-eluate -id; lane 3, viral lysate containing gp70 as positive control.

However, the fact that IC5F5 also detects non-glycosylated precursors indicates that the epitope itself is not composed of carbohydrate residues.

Epitope specificity

As shown by the Western blots both antibodies recognize the same precursor proteins $Pr65^{gag}$ and $Pr27^{gag}$. A radio-immuno competition assay was undertaken to define the epitope specificity. Figure 7 indicates that auto-competition could be provoked by pre-incubation with dilutions of identical, unlabelled MAbs. In the case of IC5F5, 85% loss of radioactivity was achieved by auto-competition (a), whereas with 4D2B4 this could be replaced for 60% of its maximal binding (b). Pre-incubation with the alternative unlabelled antibody did not inhibit binding of the other labelled antibody. Apparently both MAbs are directed against 2 different antigens or 2 different epitopes on the same antigen.

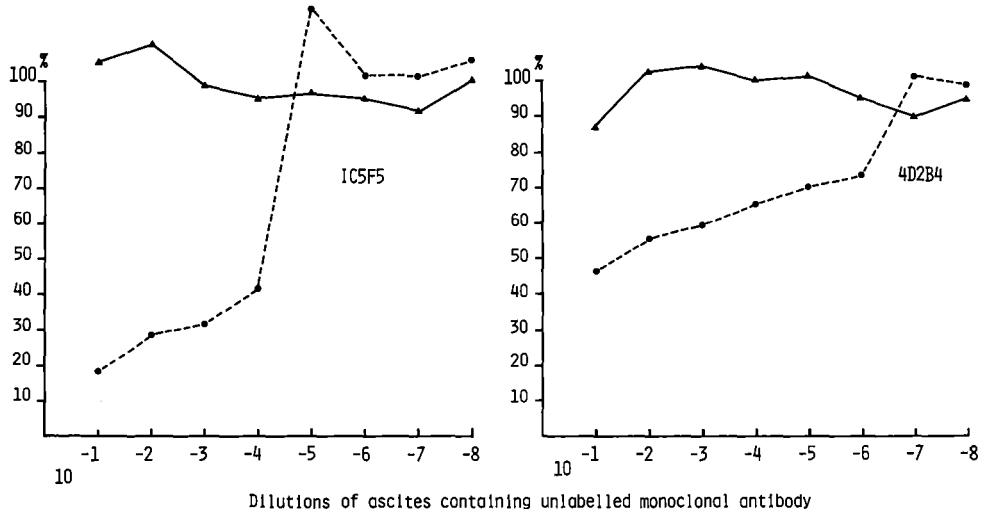

Fig. 7. Competition of unlabelled IC5F5 (left) and 4D2B4 (right) with the same (●---●) or the other non-competitive, (▲——▲) ^{125}I-labelled MAb.

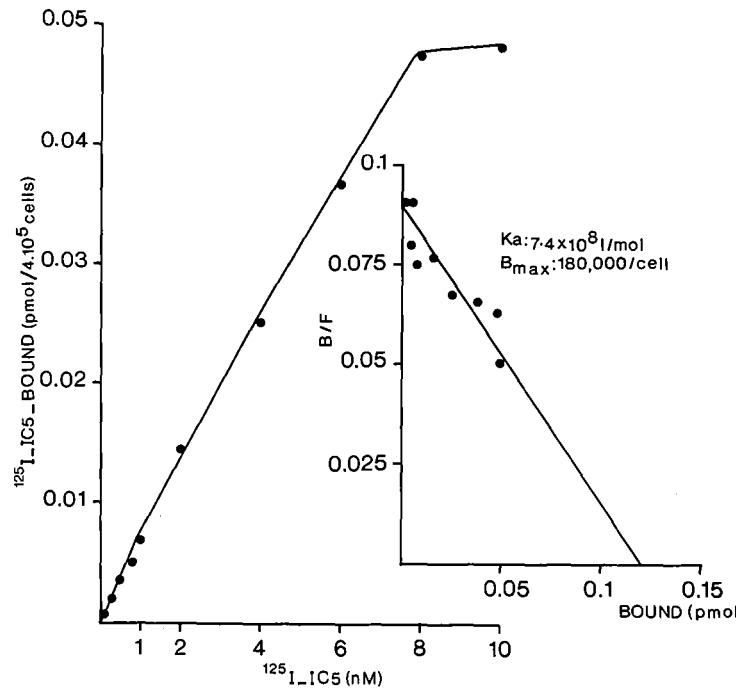

Fig. 8. Determination of number of binding sites and association constant (Ka) of ^{125}I-labelled IC5F5 with Scatchard plot. B max is the number of binding sites per cell under saturating conditions.

Antigen density

One of both MAbs IC5F5 was selected for immunotherapy. Since the density of the antigen may be important for effective therapy, a Scatchard analysis was carried out which is shown in Figure 8. A specific binding of 90-95% of the ^{125}I-labelled Mab was shown. The number of binding sites was calculated to be 1.8 x 10^5/cell with an affinity constant (Ka) of 7.4 x 10^8l/mol.

DISCUSSION

When Rauscher helper virus induced myeloid leukemic cells are transplanted syngeneically in adult mice an immune response is evoked against these cells. This allows the production of MAbs from spleens of mice repeatedly injected with lysates or sublethal doses of these tumor cells.

During the last 2 decades several studies have been published on related subjects. Bianco et al. (1966) described an immune response of mice injected with X-irradiated virally induced lymphoma cells resulting in a resistance to subsequent transplants mediated by cytotoxic and virus-neutralizing antibodies. After infection, C57Bl mice initially show a progression of chronic leukemia, but this phase is followed by regression of the leukemia and recovery of the animal. Young BALB/c mice are susceptible to Mo-MuLV, while older mice are not (Fefer et al., 1967). An antigen called FMR, which was shared by cells infected with Friend, Moloney and Rauscher virus (Old et al., 1964), was thought to be responsible for this observation.

Some authors suggest that such antigens are only associated with tumor virus infection and are not a part of viral proteins. Alaba et al. (1979) described syngeneic antibody formation after infection of BALB/c and C57Bl mice with Rauscher virus. These antibodies were directed against non-viral proteins of 46 and 21 kDa. In a number of studies Rogers (1978, 1984) showed evidence that the FMR antigen is a glycoprotein of 175 kDa, which can be physically separated from viral proteins. Immunization with gp175 prevents the outgrowth of transplanted tumor cells by the formation of cytotoxic antibodies.

Other authors stress the importance of viral proteins in causing the induction of anti-FMR antibodies (Strand et al., 1974; Nowinski et al., 1978; Chesebro et al., 1981), gp70 and p15 being the main candidates.

In the present study 2 MAbs are described reacting with p12 in purified virus and with proteins of 65 and 27 kDa, which are precursors of p12 (Van Zaane et al., 1976; Naso et al., 1979; Yoshinaka et al., 1980).

A syngeneic Mab reacting with p12 was prepared by Chesebro et al. (1983) from mice recovering from Friend leukemia. Other investigators have isolated anti-p12 MAbs following xenogeneic immunization (Gambke et al., 1984; Pillemer et al., 1986). These MAbs had anti-FMR activity. Our 2 MAbs had affinity only to the related Friend virus, but not to Moloney or AKR virus.

In RMB-1 cells a 50, 29 and (inconsistently) a 26 kDa band bind to IC5F5, but no binding to Pr65 was observed. The 50 kDa protein is a glycoprotein. The absence of p12 in RMB-1 cells (Fig. 5) is probably due to the fact that these cells do not produce virus.

Some authors suggest that high-molecular-weight glycoproteins $gp95^{gag}$ and $gp85^{gag}$ synthesized from a Pr75 (Tung et al., 1977) can be converted into a number of glycoproteins ranging from gp55 to gp27 (Fibbing et al., 1981) including gp45 and gp40 (Naso et al., 1983; Pillemer et al., 1986).

Immuno-electron microscopy indicates that the antigen to which IC5F5 and 4D2B4 are directed is localized in the membrane of RMB-1 cells. Scatchard analysis showed a high association constant and a number of 1.8×10^5 binding sites. Since the MAbs, bind to aberrant processed (glyco)proteins sharing p12 epitopes, it is likely that these proteins are incorporated in the cell membrane. In virus producing RLD-1 cells, IC5F5 binds especially to the budding particles, perhaps due to the presence of p12 or its precursor proteins. These findings are at variance to those of Schwarz et al. (1976) who found abundant presence of p12 in the cellular membrane of C-virus producing cells, but not on membranes of non-producing cells.

Only a few reports deal with the establishment of syngeneic MAbs. Kennel et al. (1985) have reported the isolation of MAbs directed against a non-viral 65 kDa protein in Moloney virus-induced sarcomas. Syngeneic MAbs are also made from animals with non-virally induced tumors, as for instance in animals bearing a Lewis lung carcinoma (Midoux et al., 1984) or in mice injected with the B16 melanoma (Vollmers et al., 1985).

Due to their high specificity, such syngeneic MAbs are excellent tools for analysis of the mechanisms underlying immunological destruction of tumor cells.

Ultimately, they offer the possibility of direct immunotherapy without the occurrence of strong anti-antibody reactions.

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CHAPTER III

THE DETECTION OF VIRALLY INDUCED TUMORS BY ^{131}I- AND ^{125}I-LABELLED SYNGENEIC MONOCLONAL ANTIBODIES

Keyword: Tumor targeting

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SUMMARY

The binding of the syngeneic monoclonal antibodies IC5F5 and 4D2B4 to Rauscher virus induced myeloid leukemic (RMB-1) cells was analyzed *in vivo* in tumor bearing BALB/c mice.

To verify if these antibodies bind specifically to RMB-1 cells, purified antibodies were iodinated with the isotopes ^{125}I and ^{131}I. Mice previously inoculated with tumor cells were injected with these labelled monoclonal antibodies and the plasma clearance as well as the tissue distribution was determined.

The clearance in tumor bearing animals was faster than in control mice. The tissue distribution was corrected for non-specific accumulation by scoring for an unrelated antibody. Calculation of a localization index showed that IC5F5 binds at least 4.5 times more specifically to tumor cells than to other tissues.

A preferential localization of radioactivity in subcutaneous tumor tissue was seen in the scanning of animals injected with ^{131}I-labelled antibodies.

The most direct proof of specific binding was observed in autoradiograms of animals treated with ^{125}I-labelled antibodies.

Small islands of tumor cells in the livers of mice inoculated intravenously both tumor cells had a high density of grains compared to other tissues and also compared to tumor cells in mice treated with unrelated monoclonal antibodies.

These results show an efficient targeting of these monoclonal antibodies and make an immunotherapy of these myeloid leukemic cells possible.

INTRODUCTION

The development of the hybridoma technology by Köhler and Milstein (21) has resulted in the production of monoclonal antibodies against assorted tumor cells. These antibodies have been used both for the detection and therapy of cancer. For instance the use of highly sensitive autoradiographic techniques allows the detection of radiolabelled monoclonal antibodies in tumor tissue (13,24,30). Also, by in vivo scanning tumor cells may be localized by monoclonal antibodies bound to radioactive isotopes (1,4,33).

For the treatment of tumors mostly heterologous or allogeneic monoclonal antibodies have been used with the drawback that these antibodies were not completely tumor specific and were directed against differentiation antigens. For this reason its application is mainly restricted to antigens expressed in large amounts by certain tumors such as the carcino-embryonic antigen (CEA), human chorionic gonadotrophin and α-fetoprotein (14). Moreover, these xenogeneic antibodies evoke an anti-antibody formation, which may interfere with prolonged immunotherapy.

In a previous study we have described the establishment of two syngeneic monoclonal antibodies directed against a permanent growing cell line derived from a Rauscher virus (R-MuLV) induced murine myeloid leukemia. These monoclonal antibodies proved to be specific for virally transformed leukemic cell lines and are able to identify a viral antigen with a molecular weight of 12×10^3 daltons and its precursor proteins.

In this study we report the labelling of one of these monoclonal antibodies with ^{125}I and its injection in tumor bearing mice to detect these tumors by scanning. The specific binding was analysed quantitatively in these mice by measuring the radioactivity in the tumor and various organs. In a second experiment the specific localization of ^{125}I-labelled monoclonal antibodies was demonstrated in mice bearing solid and disseminated tumors by autoradiography.

MATERIALS AND METHODS

Mice and tumors

Male and female BALB/c mice, 6 weeks old were obtained from TNO Zeist (The Netherlands) and kept under routine conditions. Subcutaneous tumors were induced after injection of 10^7 myeloid leukemic cells (RMB-1) which were induced by Rauscher Murine leukemia virus (R-MuLV) and which grow permanently *in vivo* and *in vitro* (10,11). Animals with disseminated tumor foci in lungs and hemopoietic organs were obtained three days after intravenous (i.v.) injection of 10^7 RMB-1 cells.

Monoclonal antibodies

In this study two monoclonal antibodies i.e. IC5F5 and 4D2B4 were used of the IgG2a and IgG2b subclass, which are specific for RMB-1 cells. Details concerning the generation and purification have been described previously (5a). As control IgG2a was used, isolated from ascites fluid of BALB/c mice bearing the hybridoma 4E3, which does not bind to RMB-1 cells.

Iodination of antibodies

A. Iodination of monoclonal antibody with ^{131}I was carried out according to a modification of the method described by McFarlane (26). Purified monoclonal antibodies (3 mg) in 50 µl of PBS were added to 15 µl of 1 M glycine/NaOH pH10. 25 µl of 1 M glycine/NaOH (pH10) were added to 25 µl of $Na^{131}I$ (1 mCi) dissolved in dilute NaOH (Amersham, Int. Bucks, U.K.). To this solution 10 µl of 10 mM cold ICl in PBS were added and immediately mixed with monoclonal antibody solution. After 5 min incubation at room temperature the reaction was stopped by separating monoclonal antibody and free $Na^{131}I$ on a Sephadex G50 (Pharmacia, Uppsala, Sweden) column in PBS. The fractions containing the iodinated monoclonal antibodies were pooled, and stored at 4°C. The specific activity was determined by measuring the radioactivity and protein content and was 0.6 mCi/mg. The binding of the antibody was determined under saturating conditions. Briefly, target and control cells were coated on microtiter well trays (Nunc, Denmark) and incubated with labelled antibody diluted in PBS + 2% BSA. The wells were thoroughly

washed, trays were cut out with a hot scalpel and counted separately in a LKB gammacounter. The binding ranged from 10% to 40% of the amount of added radioactivity.

B. ^{125}I-labelled monoclonal antibodies were prepared according to Bolton and Hunter (7). The ^{125}I-Bolton-Hunter reagent (1 mCi sp./act. 2200Ci/mol) was purchased from Amersham, U.K. After evaporating to dryness under nitrogen at room temperature, 100 µg of purified monoclonal antibody in 0.1 M Na phosphate buffer pH 7.4 was incubated with the reagent for four hours on ice. The unbound ^{125}I was removed by gelfiltration on a Sepharose G50 column. Purity of the labelled antibody was checked by polyacrylamide gel electrophoresis followed by autoradiography according to Laemli (22). The specific activity of the labelled monoclonal antibodies ranged from 1.5 - 2.2 µCi/µg protein. The binding of the labelled antibody was determined as described before. It was 40-50% for 4D2B4 and 45-67% for IC5F5.

Clearance of injected antibodies from the peripheral blood

Amounts varying from 1.5 to 15 µCi ^{125}I-labelled monoclonal antibody IC5F5, 4D2B4 or 4E3 in PBS were injected in the lateral tail vein of healthy and tumor bearing mice. Two drops of Lugols solution were added to 100 ml of drinking water to inhibit thyroid uptake of radioactive iodine. At various times after injection the radioactivity was measured in 1 µl serum collected from the orbital plexus. The binding of the isotope to circulating immunoglobulins was again verified by gel electrophoresis followed by autoradiography.

Scanning

Tumor bearing mice were given a single i.v. injection of 150-200 µCi ^{131}I-IC5F5 or ^{131}I-4E3 and scintigraphy was carried out 3-7 days later using a gamma scintillation camera with a pinhole collimator (PhoGamma III HP, Siemens, Gamma-sonics). For imaging mice were anaesthesized by intraperitoneal injection of 0.2 ml Hypnorm (Duphar) 1:4 diluted in PBS and put in the prone position. The data were collected in a dedicated computer system. Images were obtained in a 64 x 64 wordmatrix from the ^{131}I peak (364 KeV) at the third, fifth and seventh day.

Quantitative analysis of the antibody distribution

After the final scintigraphy all mice scanned were killed and the tumor and selected organs were excised dried and weighed. The radioactivity of each organ was measured in a gamma counter. Results were expressed as counts per minute (cpm) per milligram of dried tissue. Ratios of the radioactivity content of tumor and different organs to muscle tissue were determined since the latter can be used as a well vascularized control tissue. In addition ratios of tumor tissues to blood were determined, which allows comparison with other studies. To control aspecific distribution also ^{131}I-labelled 4E3 was injected. This antibody does not bind to RMB-1 tumor cells *in vitro*. The results are expressed graphically.

The specific distribution was corrected for aspecific accumulation of labelled monoclonal antibodies in tumor tissue by calculating the localization index (LI) according to Moshakis et al. (28).

$$LI = \frac{\text{counts/mg tumor IC5F5}}{\text{counts/mg muscle IC5F5}} \times \frac{\text{counts/mg muscle 4E3}}{\text{counts/mg tumor 4E3}}$$

Autoradiography

Cytopreparations of RMB-1 cells were first incubated *in vitro* with 1 µCi ^{125}I-labelled monoclonal antibody and analysed autoradiographically to verify binding. Animals were injected with 15 µCi (10 µg) ^{125}I-IC5F5 which were inoculated intravenously with 10^7 RMB-1 cells three days before or subcutaneously 2 weeks earlier. As control 25 µCi (12 µg) ^{125}I-4E3 was injected. Seventy-two hours after injection mice were killed by cervical dislocation. Tumors and selected organs were removed, fixed in 10% buffered formaline and embedded in paraffin. Sections were cut, dewaxed and dipped at 45°C in a Kodak NTB2 or Ilford G5 photo-emulsion 1:1 diluted with distilled water. They were exposed in light-tight boxes at 4°C for 2-8 weeks. After development the sections were counterstained with hematoxylin-eosine.

Sections of paraffin blocks were used both for autoradiography and for immunohistochemical staining using a two steps immunoperoxidase staining with goat anti-mouse IgG labelled with horseradish peroxidase. Sections were also treated with reticulin and elastic tissue stains for detection of blood vessels.

RESULTS

Scanning and in vivo distribution

Optimal imaging of tumors in IC5F5 treated mice was obtained 5-7 days after injection of 200 µCi ^{131}I-labelled antibody as shown in Figure 1.

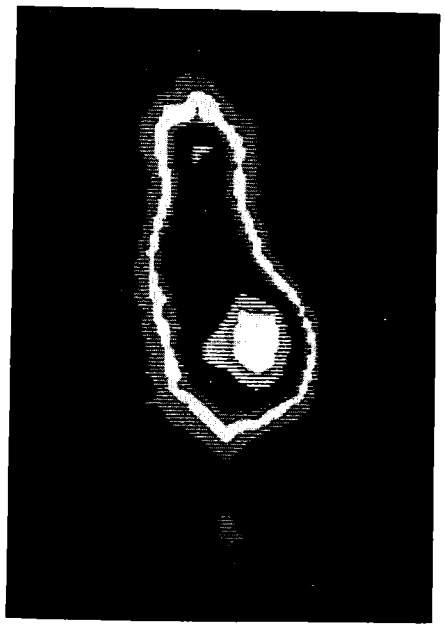

Fig. 1. Radioscintigrapy of tumor bearing mouse 7 days after i.v. injection of 200 µCi of ^{131}I-labelled IC5F5. The tumor is localized subcutaneously at the right site. Contours of the mouse are marked with a radioactive cobalt pen.

In the control group treated with 200 µCi ^{131}I-4E3, which does not bind to RMB-1 cells, no accumulation in tumor tissue was observed. Two mice in this group, bearing large, partly necrotic tumors (weight 2-2.5 gr) also had imaging of their tumors, but in these cases, equivalent activity was also present in the upper abdominal region.

After scanning the animals were killed and selected organs and tumors were excised, weighed after drying and radioactivity was measured. The average fraction of injected labelled specific monoclonal antibodies recovered from 1 gram tumor tissue was 10.6% after 7 days. For non-specific antibodies this fraction was 2.9%. The results of the measurements of radioactivity expressed as ratios of specific activity in tumor and some other organs to nonspecific activity in muscle are shown in Figure 2.

In all tumor bearing animals treated with labelled specific antibodies the accumulation was greater in tumors than in other tissues resulting in higher tumor to muscle ratios. However, kidney and heart tissue also accumulate considerable amounts. In most tumor bearing animals treated with non-specific antibodies, the accumulation in tumor tissue was greater than in other organs in only 1 of the 5 animals.

The LI calculated according to Moshakis et al. (28) was 4.44, when tumor and muscle tissue are compared, whereas it was 1.83 in the comparison of kidney to muscle and 0.99 in case of spleen to muscle. Since the tumor consists of leukemic cells, the spleen/muscle ratio was used as reference. This implies that the LI for tumor tissue was 4.5 times greater than for spleen cells. Specific binding of IC5F5 antibody to tumor was therefore evident in contrast to the binding of non-specific ^{131}I-IgG2a. When tumor to blood ratios were determined an average of 5.6 was obtained for tumor bearing mice injected with ^{131}I-labeled IC5F5 and a ratio of 0.48 for tumor bearing mice treated with ^{131}I-4E3. Calculation of the labelling index showed a corrected value of 11.7, which is two times higher than for tumor to muscle ratios.

Clearance

Mice with extended disseminated tumors and control mice were given 15 µCi (10 µg) ^{125}I-IC5F5, 24 µCi ^{125}I-4D2B4 (11 µg) or 15 µCi (7 µg) ^{125}I-4E3. Simultaneously, mice bearing subcutaneous tumors were given 15 µCi ^{125}I-IC5F5.

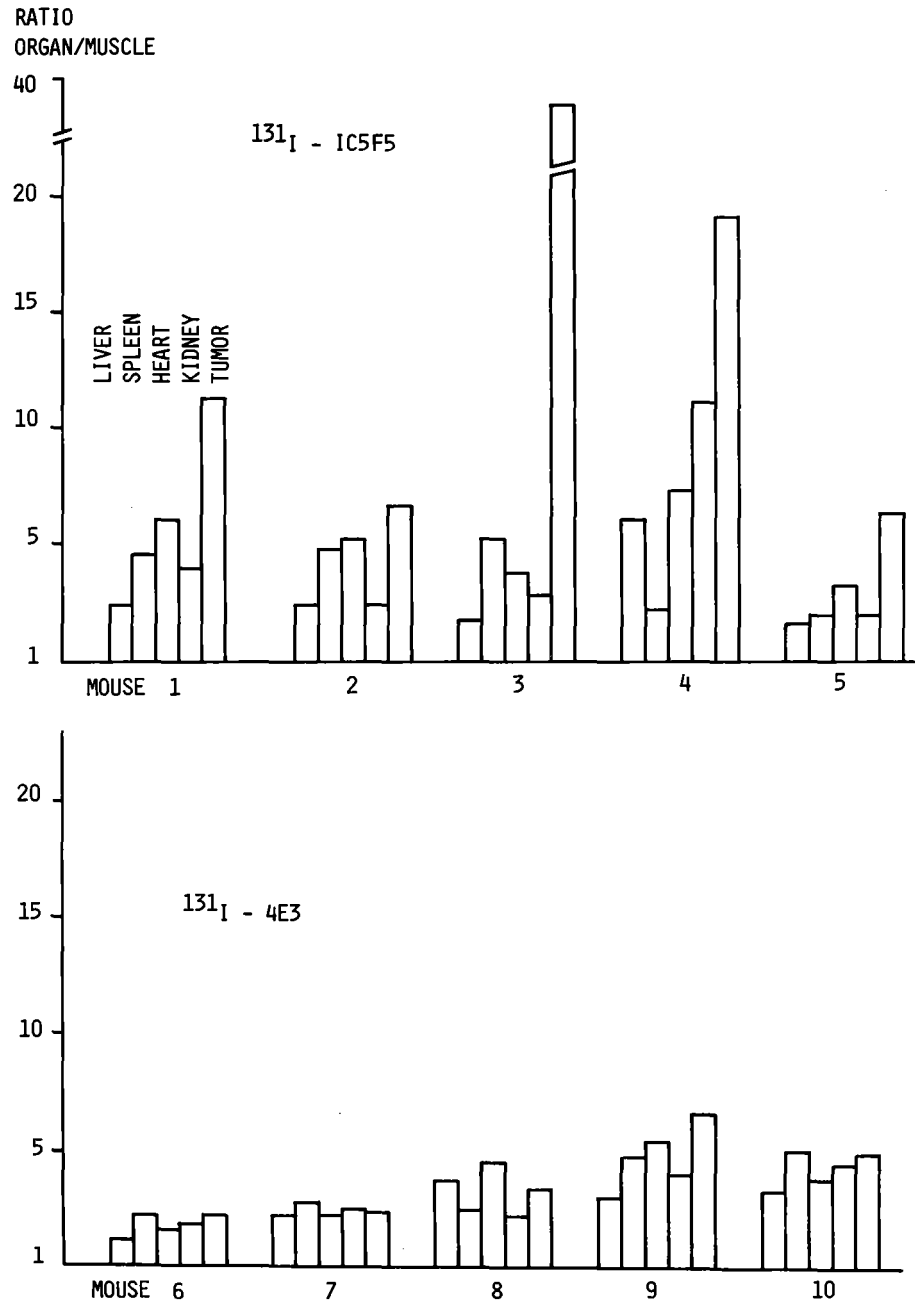

Fig. 2. Ratios of amount of radioactivity of different organs and tumor tissues taken 7 days after i.v. injection of 200 μCi ^{131}I IC5F5. For comparison tumor bearing mice are injected with 200 μCi ^{131}I-labelled 4E3.

Table 1 Percentage of residual serum radioactivity one hour after injection.

Antibody	Mice with subcutaneous tumors %	Mice with disseminated tumors %	Control mice %
^{125}I-IC5F5 (15 µCi)	38.8 ± 4.7	26.2 ± 7.0	41.0 ± 5.9
^{125}I-4D2B4 (24 µCi)	n.t.	20.7 ± 6.6	36.2 ± 4.9
^{125}I-4E3 (15 µCi)	n.t.	49.8 ± 1.3	56.5 ± 6.1

As shown in table 1 both specific monoclonal antibodies IC5F5 and 4D2B4 had a rapid decline of serum radioactivity in the first hour after injection in mice having disseminated tumors, which was faster than in control mice. This more rapid clearance continued in the following 24 hours (Fig. 3a and b). With control antibody 4E3 clearance was also rapid, but no striking differences were observed between the two groups (Fig. 3c). When animals bearing subcutaneous tumors were compared with control mice, a difference in initial clearance was less obvious (table 1, Fig. 3a). Equal patterns were seen using lower doses until 1.5 µCi (1 µg) IC5F5 (data not shown).

Autoradiography

RMB-1 cells incubated with 1 µCi ^{125}I-IC5F5 in vitro had specific affinity in the autoradiograms. Normal thymocytes did not bind IC5F5 (not shown). In vivo targeting to RMB-1 cells was carried out in tumor bearing mice after i.v. injection of 15 µCi ^{125}I-labelled antibodies followed by autoradiographic analysis. Figure 4 demonstrates foci of labelled RMB-1 tumor cells in the liver (a), spleen (b) and lung (c) of mice after i.v. injection with ^{125}I-IC5F5. Irregular foci of tumor cells were found throughout the liver parenchyma and small clusters of RMB-1 cells were detected underneath the spleen capsule. Single cells or very small clusters were seen in the capillaries of the alveolar septae of the lung. On all these spots high grain densities were present in the autoradiograms, whereas few grains were seen in

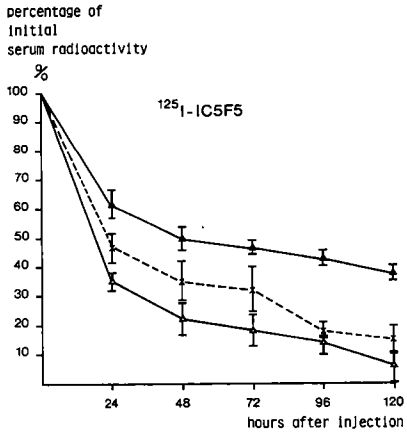

Fig. 3a. Clearance of i.v. injected ^{125}I-labelled IC5F5 from serum of mice with disseminated (Δ——Δ) subcutaneous (X---X) RMB-1 tumors and control mice (▲——▲). n = 4 for each group.

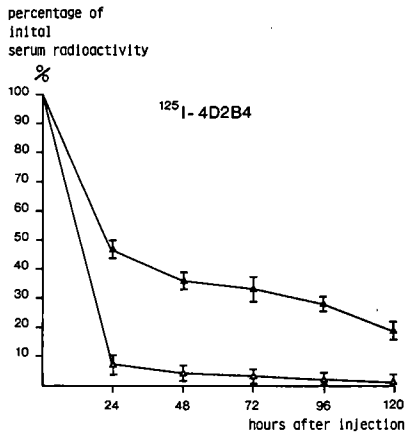

Fig. 3b. id- ^{125}I-labelled 4D2B4.

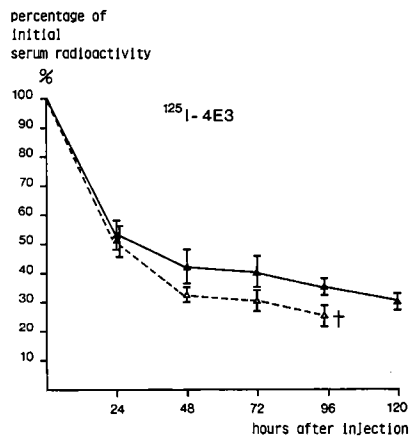

Fig. 3c. Id- ^{125}I-labelled non-binding 4E3. (Δ---Δ) mice with disseminated tumors; (▲——▲) control mice.

adjacent tissues. High grain densities were also seen in the periphery of several lymphfollicles in the spleen (not shown).

Animals treated with ^{125}I-4D2B4 had a similar grain distribution, but with less contrast. In these autoradiograms the tumor mass in the hemopoietic organs was more extensive. However, in spite of the presence of grains in surrounding well-vascularized areas, most grains were localized in tumor islands. A striking accumulation was seen in the periphery of lymph follicles of the spleen as noted for IC5F5.

In contrast to both monoclonal antibodies, the distribution of the non-specific ^{125}I-IgG2a was random throughout the different organs and is restricted predominantly to the vasculature. High grain densities were particularly found in liver sinusoids (Fig. 4e) and vascular beds of lung and kidney. No specific binding to tumor cells was noted.

Histological examination of subcutaneous tumors of animals injected with ^{125}I-IC5F5 (Fig. 4d) showed high grain densities particularly in the well-vascularized peripheral parts of the tumor. Also tumor parts with intercellular edema had grains in a narrow zone around the cells, whereas, in compact areas, only minimal grain deposition was observed. Staining of elastic and reticulin fibers showed that vascularization in these areas was poor.

The identity of RMB-1 cells was confirmed by immunoperoxidase staining using the same monoclonal antibodies. Control sections of mice treated with non-specific IgG2a did not stain these tumor cells.

DISCUSSION

The radioimmune detection of a tumor is dependent on several factors i.e. 1) The specificity of the antigens to which the antibodies are directed and their concentration on individual tumor cells. 2) The efficiency of the labelling procedure and the specific biological activity of the labelled antibody. 3) The route of administration by which the labelled antibodies reach the tumor cells and 4) A sufficient clearance of unbound labelled

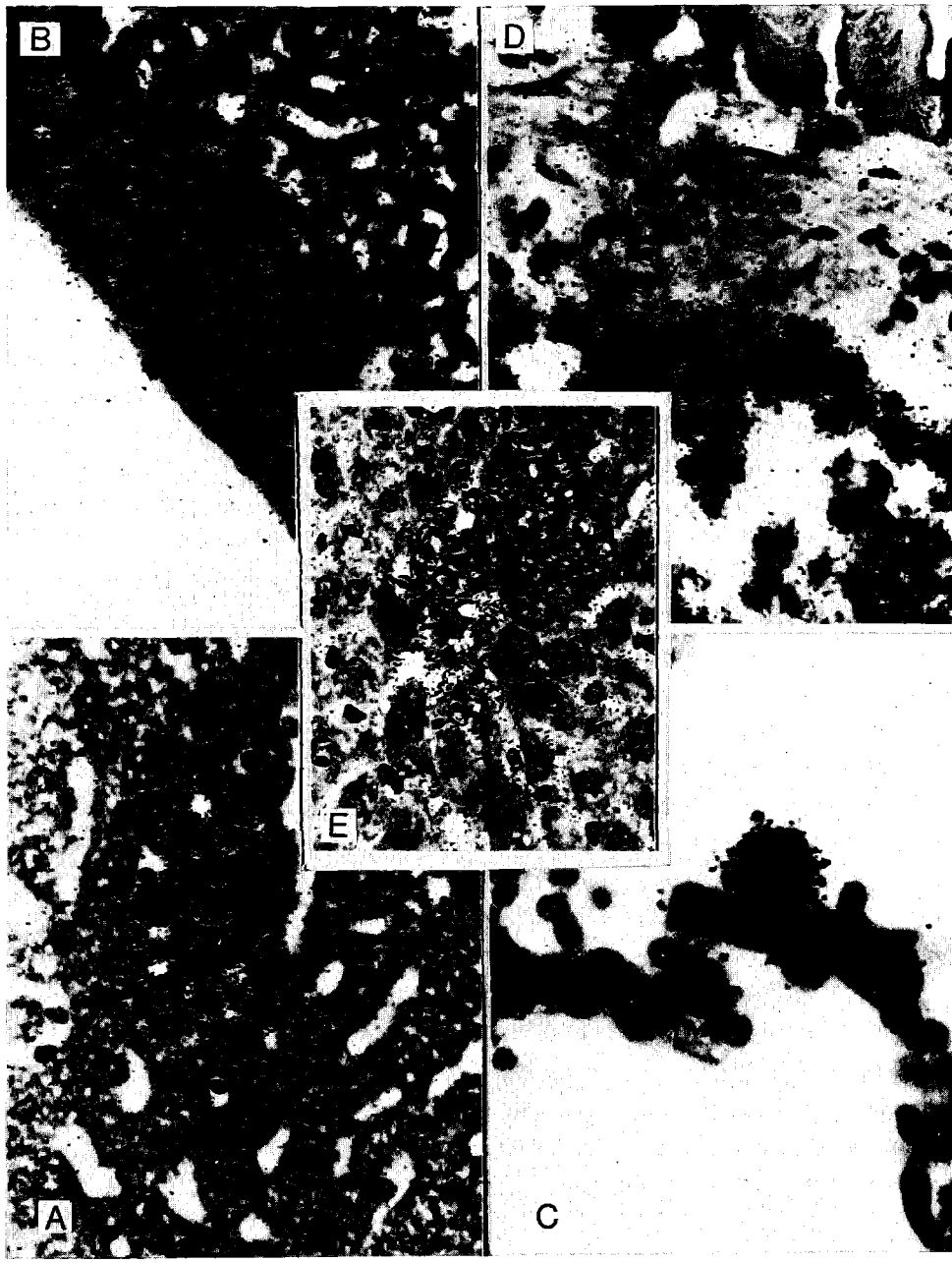

Fig. 4. Autoradiography of RMB-1 metastases in liver (a), spleen (b), lung (c), and of subcutaneous RMB-1 tumor (d) 3 days after injection of 15 μCi ^{125}I-labelled IC5F5. Liver with disseminated tumor cells of mouse injected with ^{125}I-labelled control antibody 4E3 (e). magn. 380x

antibodies to ensure detection of specifically bound antibodies. Each of these factors is discussed below.

Item 1. There is scepticism about the existence of tumor specific antigens and most authors consider these antigens at least in human cancers as onco-foetal antigens (15) such as the CEA and α-fetoprotein (19). However, in virally transformed cells, they may be related to viral proteins, to viral encoded or induced transforming proteins (35). In that sense these antigens may be considered as tumor specific.

The monoclonal antibodies used in this study as seen with ELISA and Western blotting techniques reacted extensively with Rauscher virus-induced myeloid leukemic cells and bond to the viral proteins expressed on these cells, but did not react with uninfected control cells (5a). This ensures specific detection of leukemic cells, injected in non-infected animals.

Item 2. The purification and labelling methods used did result in an electrophoretically pure antibody with a specific activity of 2.2 µCi/µg and a binding activity up to 67% of the labelled antibody added.

Item 3. In this study it has been shown that *in vivo* injected myeloid leukemic cells home in several organs with a preference for hemopoietic organs. Histologically, foci of tumor cells can be easily detected in the normal tissues, often displaying an intimate relationship to the vasculature. The i.v. administration of labelled antibodies, therefore, ensures ready accessibility to tumor cells.

Item 4. Clearance of labelled antibodies from the peripheral blood is dependent on metabolic degradation, excretion mechanisms and the binding to tumor cells (31). In this study a rapid clearance differential was found in mice with disseminated tumors as compared to controls, whereas less difference was obtained for animals bearing subcutaneous tumors.

Successfull imaging of s.c. tumors was possible from the 5th day after injection of labelled antibodies. The lowest background was observed on day seven, which is in accordance with the clearance curves and radioactivity measurements of the tumor and in other tissues.

Such retarded but specific accumulation has also been reported after scanning of a number of solid tumor bearing (nude) mice treated with monoclonal antibodies against an oncofetal marker such as CEA (9,30), melanoma-specific antigen (18), antibody directed to a mouse teratocarcinoma (23) or with antibodies recognizing a hepatocarcinoma-related antigen in guinea pigs (6). These results are in contrast to those of Scheinberg and Strand (33) using monoclonal antibodies directed against the viral glycoprotein gp70 detecting leukemic spleen cells and to data of Shah (34), who used antibodies which recognized human breast and colon tumor cells which were grafted directly into spleens. They reported excellent images within 24 hours after injection. This discrepancy may be due to the greater accessibility of these tumor cells within highly vascularized organs. This might also explain the controversial results concerning the plasma clearances in animals bearing subcutaneous tumors (27) and in animals with splenic tumor cells (31). This is supported by the studies of Sands et al. (32) who measured the blood flow in identical tumors localized either s.c. or under the renal capsule.

The positive imaging of tumors treated with non-specific labelled monoclonal antibody may be explained by the nonspecific accumulations in the intercellular space as described by Bale (2). This mechanism is probably less important in highly vascularized or small tumors with a relatively small extravascular space. The specific accumulations of tumor specific monoclonal antibodies is corrected by the nonspecific distribution of monoclonal antibodies (4,28). In this study this correction results in a 4.5 fold higher LI value for tumor cells as compared to spleen cells and a 11.7 value when tumor tissue to blood is compared. Literature data show that the fraction of labelled monoclonal antibody recovered and the tumor to blood and tumor to muscle ratios are dependent on the amount of radioactivity injected, the type of monoclonal antibody and the time of measurement. Our tumor to blood ratios lie in the same range as those of Fand et al. (13) and are about two times higher than those of Ballou et al. (3), Midoux et al. (27) and Buchegger et al. (9), but are much lower than the ratios reported by Bernhard

et al. (6). In contrast with these results Pimm et al. (13) have reported tumor to blood ratios smaller than 1.0. However, in the studies mentioned other correction methods were applied for nonspecific binding. Others have applied subtraction methods for correction of nonspecific binding using two different isotopes, one of which is bound to a nonspecific antibody (3,14).

To ensure a rapid clearance and to prevent Fc binding, some authors have used F(ab)2 fragments instead of whole antibodies (9,16,25). Although this method gives lesser background, doubts exist about its efficiency (8). In this study we used intact antibodies which also gave satisfactory results.

Autoradiographic techniques in s.c. tumors and lymph node metastases have also been carried out by several authors (24,29,30,32) as well as immunoperoxidase techniques (12,20). Nearly all authors found preferential localization in the periphery of tumors which usually is the most vascular. Our results confirm this. In the visceral tumors, however, grain density was inversely related to increasing tumor diameter. This may be explained by excess of antigen and concomittant depletion of labelled antibodies or by a lower penetration of these antibodies.

In contrast to the use of allogeneic monoclonal antibodies, the use of syngeneic monoclonal antibodies in specific targeting by pure syngeneic monoclonal antibodies made from spleens of tumor bearing animals has only rarely been described (17). This is due to the fact that not all tumors evoke an host immune response. The value of syngeneic antibodies lies therefore in their tumor specificity. This allows detection of metastases and circumvents an anti-antibody response in repeatedly treated animals (5b). Such a system is an excellent model for further exploration of the basic mechanisms in monoclonal antibody immunotherapy.

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CHAPTER IV

USE OF SYNGENEIC MONOCLONAL ANTIBODIES IN THE THERAPY OF DISSEMINATED MYELOID LEUKEMIC CELLS

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SUMMARY

A syngeneic monoclonal antibody (MAb)(IC5F5) was successfully used in the immunotherapy of Rauscher virus-induced myeloid leukemic RMB-1 cells. It is directed to a virus encoded, but aberrantly processed protein, which is expressed on the cell membrane. When applied *in vivo*, it binds only to RMB-1 tumor cells. BALB/c mice were inoculated i.p. or i.v. with 10^7 RMB-1 cells and died within 2-3 weeks due to increasing tumor load. Mice inoculated i.p. were completely cured by daily injections of ascites containing IC5F5. Disseminated tumor cells in liver and hemopoietic organs were observed after i.v. inoculation. Daily treatment with MAbs resulted in survival beyond 90 days. No antigenic modulation was observed when tumor tissue was analysed 2-10 days after treatment. Treatment was successful even when therapy was postponed until day 5 following inoculation of tumor cells. When the number of ascites injections was reduced, survival was identical to that observed among repeatedly treated mice. Ten- and 100-fold dilution of ascites fluid diminished the number of survivors, but still resulted in a median survival time of 38 and 20 days, respectively, as compared to 14 days for untreated mice.

INTRODUCTION

Immunotherapy using conventional polyclonal antibodies has been successful in virally induced murine leukemias (Schäfer et al., 1976; Collins et al., 1978) as well as in some xenografted human neoplasms (Rosenberg and Terry, 1977). The introduction of MAbs (Köhler and Milstein, 1975) has greatly increased interest in possible serotherapy of malignant tumors. For instance, in murine models, inhibition of tumor growth has been shown to occur with allogeneic MAbs in primary leukemias as well as in grafted leukemic cells (Bernstein et al., 1980; Kirch and Hämmerling, 1981; Scheinberg and Strand, 1982; Badger et al., 1986). Human tumors transplanted into nude mice have also been successfully treated in this way (Koprowski et al., 1978; Herlyn et al., 1985).

Pure syngeneic MAbs have only occasionally been used for such treatment. These were obtained as fusion products of spleen cells from immunized animals with myeloma cells bearing the same genotype (Herlyn et al., 1980; Kennel et al., 1983, 1985).

Two syngeneic MAbs have been characterized, which are directed to Rauscher virus-transformed myeloid leukemic cells (Berends et al., 1988a). Both bind to viral proteins localized in the cell membrane. Binding to tumor cells *in vivo* has been evidenced by ^{125}I-labelling and autoradiography.

We now report the therapeutic effects of MAb IC5F5, using different experimental conditions. This MAb is of the IgG2a subclass. Most previous experimental studies have used one single focus (usually s.c. tumor) in order to test the results of treatment. In contrast, our work studies the effect of immunotherapy on disseminated tumors and as such it more accurately reflects metastasized cancers, which are usually difficult to cure.

MATERIALS AND METHODS

Cell line

We have used the myeloid cell line RMB-1, which is induced by Rauscher virus to grow permanently *in vitro* and *in vivo* (De Both et al., 1981; Delwel et al., 1987). Cells were cultured in RPMI 1640 + 10% fetal calf serum (Seromed, Miles, Brussels)

Mice and tumor

Male and female BALB/c mice, 8-12 weeks old, were obtained from TNO, Zeist (The Netherlands). Intra-abdominal and disseminated tumors were obtained by i.p. or i.v injection of 10^7 RMB-1 cells.

Monoclonal antibody

Ascites fluid containing MAb IC5F5 was obtained after injection of 15 x 10^6 hybridoma cells i.p. in BALB/c mice pretreated 10-21 days earlier with 0.5 ml Pristane (Janssen, Beerse, Belgium). Ascites fluid of mice bearing the non-binding hybridoma 4E3 was used as control.

In vitro modulation

RMB-1 cells were co-cultured with or without 1% IC5F5 or 4E3 ascites. After 2 days the cultures were centrifuged and re-fed in culture medium with or without ascites. The number of cultured cells was counted daily. Some cultures were measured for expression of the IC5F5 antigen by flow cytometry, while others were incubated for 2 more days and then measured for antigen expression. Flow cytometric measurement of IC5F5 antigen expression was carried out as follows: 10^6 cultured RMB-1 cells were washed twice with Hanks' balanced salt solution (HBSS) containing 5% FCS and 0.02% sodium azide. They were then incubated for 45 min on ice in 50 µl IC5F5 or 4E3 ascites, both diluted 1:1,000 in HBSS. The cells were again washed with HBSS, then labelled for 30 min with goat anti-mouse IgG2a/fluorescein isothiocyanate (GAM/FITC; Nordic, Tilburg, The Netherlands), diluted 1:50 in HBSS. After washing, the cells were resuspended and analyzed for IC5F5 antigen expression using a fluorescence activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, CA).

In vivo modulation

Tumor bearing BALB/c mice were treated with IC5F5 or with 4E3 ascites fluid as control, and killed 2, 4 and 10 days after the beginning of treatment. Organs were excised and frozen sections were either pre-incubated with IC5F5 or 4E3 ascites (1:1,000), followed by incubation with goat anti-mouse IgG labelled with peroxidase (GAM/PO), or stained directly with GAM/PO, to detect residual previously injected mouse MAbs.

Therapy experiments

In order to determine the dose of RMB-1 cells needed to kill 50% of the mice (LD 50), animal survival was determined after i.v. inoculation of 10^5 -2.10^7 RMB-1 cells into groups of 9 mice each. Animals were monitored daily to score their mortality.

Treatment of intra-peritoneal and disseminated tumors

Mice were inoculated with 10^7 RMB-1 cells either i.p. or i.v. Immunotherapy was started with 0.1 - 0.2 ml ascites containing MAb IC5F5, to which 0.05 ml fresh normal rabbit serum was added. The antibody titer in different ascites batches was tested using an enzyme-linked immunosorbent assay (ELISA). Batches exhibiting comparable activity were mixed together to obtain equivalent preparations. Typical preparations had a protein content of about 50 mg/ml, which is equivalent to 2 mg Sepharose protein A-purified MAb. This produces an extinction of the positive signal in an ELISA at a dilution of 10^{-5}. Control animals received phosphate buffered saline (PBS) or ascites of BALB/c mice inoculated i.p. with the non-binding hybridoma 4E3. Animals were monitored daily for mortality. The results are expressed graphically as percentage of survivors against time. The median (MST) as well as the average survival time $\pm$ the standard deviation (SD) were scored.

In order to determine the extent of tumor growth, mice were killed after different periods of time without treatment and their tissues were embedded for histological examination.

Statistics

Survival curves of treated and untreated groups as observed on the 60th day were tested for similarity by Breslow's version of the Wilcoxon test, using program 1L of the BMDP statistical

software package (Dixon, 1983). The statistics tested correspond to a chi-square distributed variable with 1 degree of freedom. A probability of 1% or less ($P < 0.01$) between 2 survival curves was considered as significantly different.

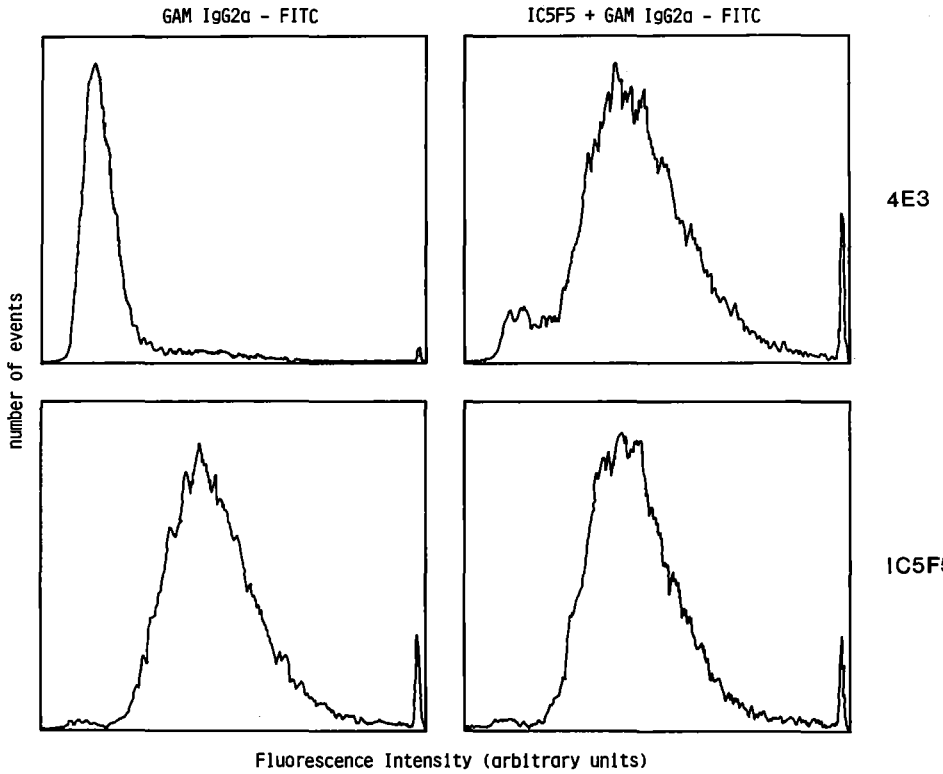

Fig. 1. Fluorescence of RMB-1 cells cultured for 2 days with or without IC5F5 ascites. Fluorescence is expressed logarithmically.

RESULTS

Absence of in vitro antigenic modulation

Antigenic modulation may influence the results of immunotherapy. We thus tested the possible occurrence of antigenic modulation *in vitro*. After culture of RMB-1 cells for different periods with a saturating amount of IC5F5 ascites, the presence of antibody and antigens was tested by indirect immunofluorescence using a FACS. Figure 1 shows that the presence of the specific antigen remained unaltered during the whole incubation period and that it was not influenced by the presence of antibody. After 2 and 4 days similar results were obtained. This indicates that shedding of the IC5F5 antigen does not occur. No difference in the number of cells was observed between cultures with and without antibody, indicating that proliferation was not inhibited.

Therapy

A. Treatment of intraperitoneal tumors

To evaluate immunotherapeutic effects of IC5F5 a series of experiments was made using mice inoculated i.p. with 10^7 RMB-1 cells 24 hr earlier. This dose kills mice within about 3 weeks. Mice inoculated with RMB-1 cells were treated i.p. with 0.1 ml IC5F5 ascites daily for 14 days. One group of animals was treated with 0.1 ml IC5F5 ascites and 0.05 ml fresh rabbit serum to verify whether therapy is improved by complement (Bernstein et al., 1980). Figure 2 shows survival after 20 days. All control mice treated with 0.1 ml PBS plus 0.05 ml rabbit serum died rapidly within this period, whereas 80-100% of the animals received no rabbit serum remained alive. The final results obtained with and without addition of complement were similar, so complement was omitted from further experiments.

B. Treatment of disseminated tumors

Since fatal intraperitoneal i.p. tumor growth was abolished by treatment with specific MAbs, the effect of this treatment was also investigated in mice inoculated i.v. with RMB-1 cells.

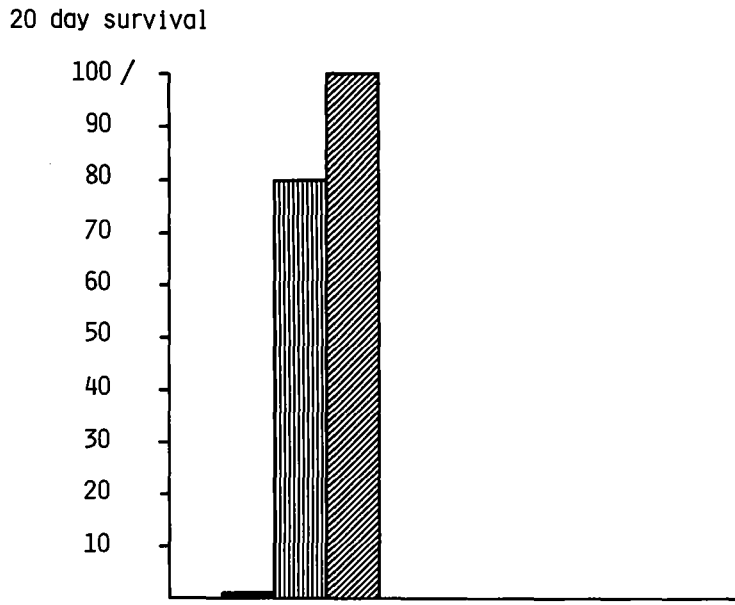

Fig. 2. Percentage of survival 20 days after i.p. inoculation of 10^7 RMB-1 cells. (▥) Mice treated i.p. with 0.1 ml IC5F5 ascites; or (▨) 0.1 ml ascites + 0.05 ml normal rabbit serum; or (■) 0.1 ml PBS + 0.05 ml normal rabbit serum. Therapy was started 24 hr after tumor cell inoculation. n = 10.

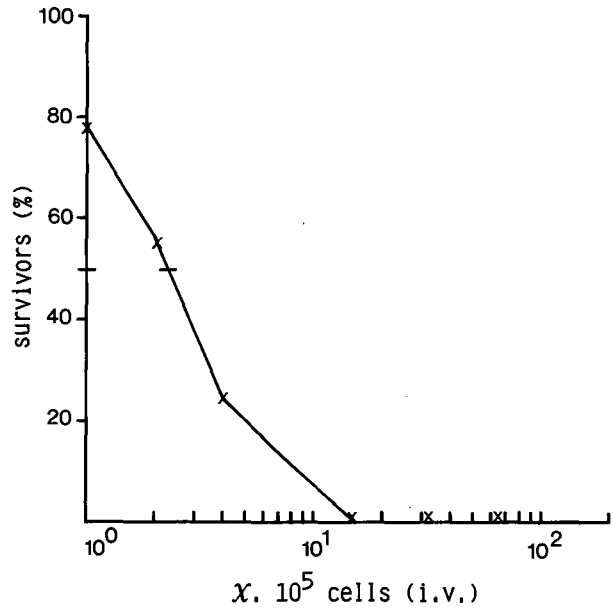

Fig. 3. Survival of mice injected i.v. with different numbers of tumor cells (plotted logarithmically). Each point represents 9 mice. Short horizontal bar indicates LD 50.

These leukemic cells home initially to the liver, spleen and bone marrow and thereafter to other organs, resulting in disseminated cancer, which rapidly kills the animal. Because this situation resembles metastatic tumor growth, we decided to use it as a model to test the immunotherapy.

First the dose capable of killing 50% of the mice (LD 50) was determined (Fig. 3). Fifty percent of the animals died after i.v. inoculation of 2-3 x 10^5 RMB-1 cells. Inoculation of 10^7 cells resulted in 100% death within 15-20 days.
However, when treated with daily injections of IC5F5 starting 24 hr after inoculation, mice showed prolonged survival. Mice treated with ascites fluid of the non-binding antibody 4E3 all died within 20 days (data not shown).

To ensure outgrowth of inoculated RMB-1 cells to micrometastases in the organs in another series of experiments we waited at least 3 days before starting the IC5F5 treatment.

C. Variation in titer of antibodies applied

Groups of 20 mice were treated daily i.v. for 10 days with undiluted IC5F5 ascites or with various dilutions (Fig. 4a). After injection of undiluted IC5F5 ascites, 70 % of the animals were cured (survival for over 90 days). Injection of a 10-fold dilution resulted in 45% cure (MST 38 days). A 100-fold dilution still resulted in a higher median survival time (MST 20 days) than that of control animals (MST 10 days). A 1,000-fold dilution, however, did not significantly alter long-term survival. Statistical analysis of survival curves of the various experimental groups compared with the survival curve of control mice revealed significant differences ($P < 0.01$). The median and average survival times, as well as the various p values are presented in Table I.

Table I Effect of antibody dose on host survival*

Antibody	Dilution	Number of survivors/ total number of mice	Median survival time (MST)	Average survival time ± SD	P value treated/ control
IC5F5	undiluted	(14/20) - 70%	60	53.3 ± 3.2	$P < 10^{-4}$
IC5F5	1:10	(9/20) - 45%	38	38.8 ± 5	$P < 10^{-4}$
IC5F5	1:100	(3/20) - 15%	20	24.9 ± 3.5	$P < 10^{-4}$
IC5F5	1:1000	(0/20) - 0%	14	14.2 ± 0.3	$P < 3.10^{-4}$
4E3	undiluted	(0/20) - 0%	10	11.3 ± 0.4	-

* measured up to 60 days after inoculation of tumor cells

D. Postponed treatment

Since prolonged survival was obtained when treatment was started at least 72 hr after inoculation, the therapy-free interval was extended to 5 or 7 days. The results are shown in Figure 4b. Survival after a 5-day interval was similar to that observed after a 3-day interval as shown in Figure 4a. However, a 7-day delay resulted in death of all treated mice. Figure 5 shows a representative example of tumor growth in the liver of untreated mice at day 5 after inoculation. Tumor foci were also found in the spleen and bone marrow and, to a lesser extent, in the lungs.

E. Single versus repeated doses

To compare the effect of a single injection of 0.2 ml undiluted IC5F5 ascites with that of repeated administration, groups of mice inoculated with 10^7 cells i.v. were treated, starting 72 hr later either once or 4 times at intervals of 4 days. Control mice received 4 injections of 0.2 ml non-binding 4E3 ascites. As shown in Figure 4c the survival is nearly identical in the 2 groups treated with specific MAb and is comparable to the results of daily treatment with undiluted IC5F5 ascites, as shown in Figure 4a. Obviously, high initial antibody

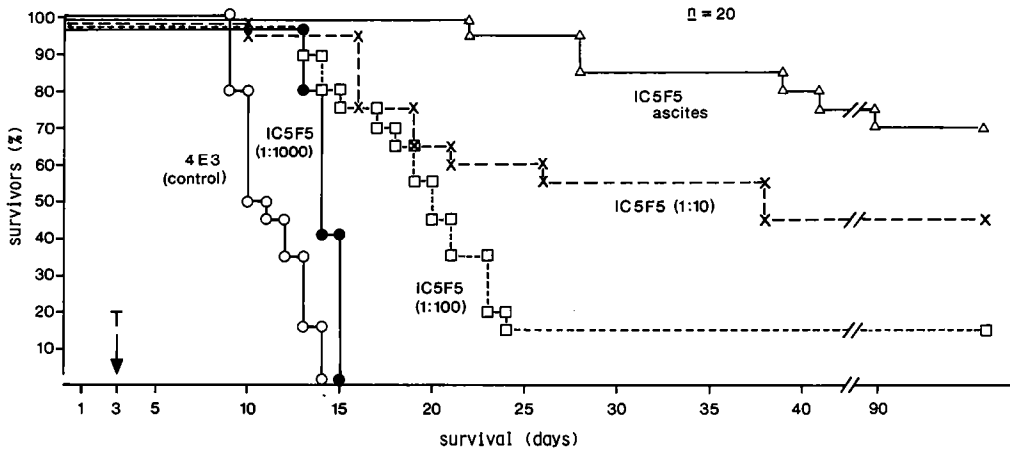

Fig. 4a. Survival of mice inoculated i.v. with 10^7 RMB-1 cells after treatment with 0.2 ml IC5F5 ascites, or with 4E3 ascites as control. Daily treatment with various dilutions of IC5F5. T = start of treatment.

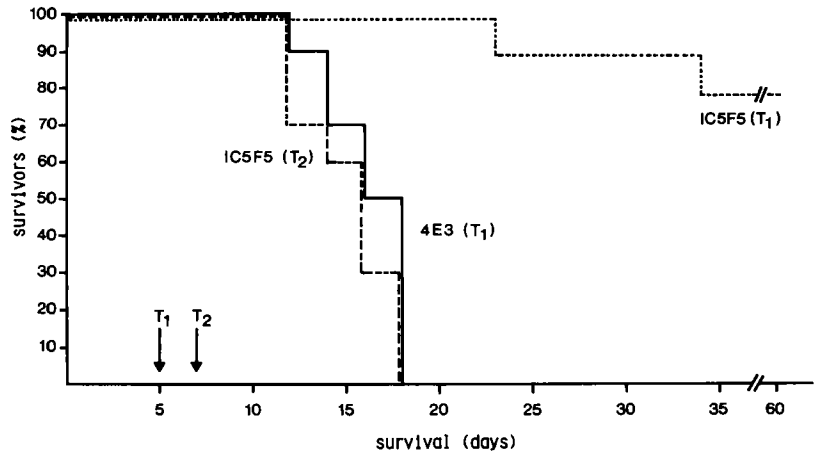

Fig. 4b. See legend to figure 4a; start of treatment postponed until day 5 (T1, n = 9) or day 7 (T2, n = 10) after inoculation.

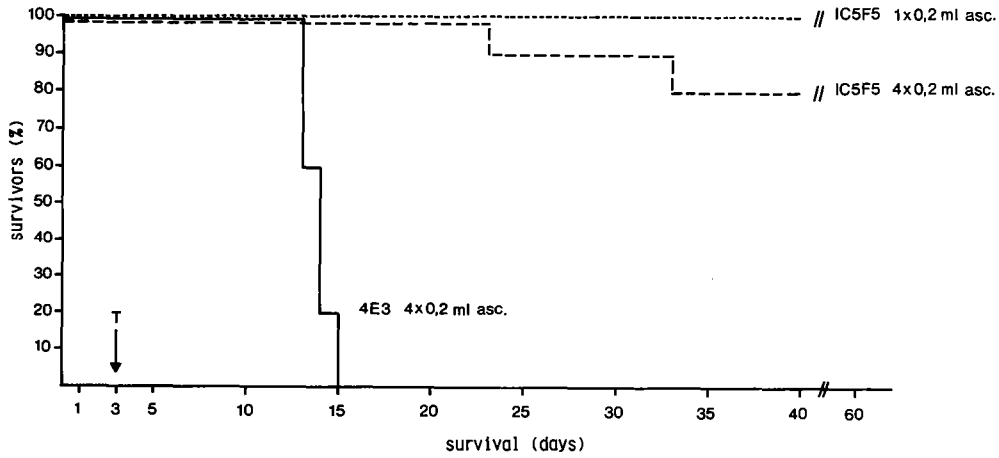

Fig. 4c. See legend to figure 4a; comparison of 4 injections at 4 day intervals with one single injection (n = 10).

levels are of the utmost importance for successful immunotherapy in this disseminated tumor model.

Absence of in vivo modulation

Modulation of the antigen recognized by IC5F5 does not take place in vitro. To exclude possible interference with treatment, modulation was also studied in vivo. Frozen sections made of livers of tumor bearing mice treated for 2, 4 and 10 days by daily i.v. injections of 0.2 ml IC5F5 or 4E3, were directly stained with GAM/PO. Other sections of the same liver were pre-incubated with IC5F5 ascites before incubation with GAM/PO. In Figure 6a a focus of tumor cells can be seen in the liver of a mouse treated with IC5F5 for 48 hr. The section is stained with GAM/PO only. The presence of antibody is shown by positive staining of the periphery of the tumor. Upon pre-incubation with MAb all the cells are stained (Fig. 6b). These data indicate that the target antigen detected by IC5F5 persists on the tumor, but that in larger foci i.v. injected antibody binds predominantly to the periphery of the tumor tissue. Sections of organs of mice treated with 4E3 did not show any staining after incubation with GAM/PO.

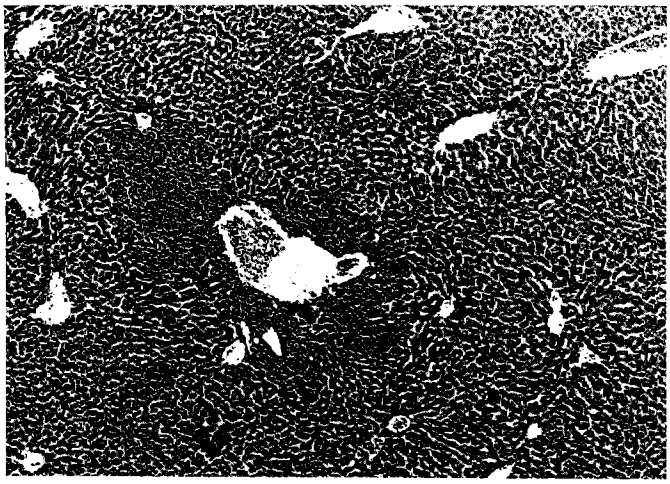

Fig. 5. Extent of tumor growth in liver 5 days after tumor cell inoculation.

a

b

Fig. 6. Peroxidase staining of serial frozen sections of liver containing disseminated tumors, treated twice with IC5F5 ascites. (a) staining with GAM/PO; (b) pre-incubation with IC5F5 followed by staining with GAM/PO.

DISCUSSION

Previous studies have demonstrated that the efficacy of cancer immunotherapy is influenced by a number of factors. One of the most important of these is the specificity of the antigens to which the (monoclonal) antibodies are directed. In the case of human tumors, antibodies against the oncofetal antigens CEA in colorectal carcinomas (Herlyn et al., 1980) or against the idiotype of B cell lymphomas (Miller et al., 1982) are sufficiently specific to allow immunotherapy to be used.

In animal models treatment often comprises antibodies which are directed to differentiation antigens, such as Thy-1.1, expressed on T cell lymphomas (Bernstein et al., 1980, Badger and Bernstein, 1983), or to tumor-associated glycosylated antigens which are not present on normal cells (North and Dean, 1983). The RMB-1 cells used in this study express viral proteins which can be considered as highly tumor-specific.

The density of the antigens seems equally important, since variants having a smaller amount of antigen are more difficult to treat (Herlyn et al., 1985). Other major drawbacks of immunotherapy are that selection of antigen-deficient populations may take place in tumor cells (Young and Hakomori, 1981) and that antigen modulation may occur (Old and Stockert, 1977; Schroff et al., 1984).

Although only a moderate number of target antigens is distributed on the cells used in our model, as shown by Scatchard analysis (Berends et al., 1988a), successful immunotherapy is nevertheless possible. No modulation occurs in our cells, either *in vitro* or *in vivo*. Apparently the viral protein to which IC5F5 is directed is consistently expressed on RMB-1 cells. Since these cells do not produce virus, no abundant circulating antigens are present in the serum of tumor-bearing animals which might block the injected MAbs.

The class and subclass of the antibody applied for therapy may be important. As pointed out by many authors the IgG2a subclass is particularly powerful in mediating the complement dependent cytotoxicity and antibody dependent cellular cytotoxi-

city of macrophages and killer cells (Koprowski et al., 1978; Steplewski et al., 1983; Adams et al., 1984; Denkers et al., 1985). This may explain our good results. In a subsequent study we will deal with the possible mechanisms underlying the efficient therapy reported here.

Other immunotherapeutic studies show that the number of tumor cells destroyed by successful treatment varies considerably. This may reflect differences in malignant potential, which depends on the take of the tumor cells, their growth properties and differences in the immune response of the host. Comparison is therefore difficult and must be related to the median survival time (MST) and the number of cells necessary to cause tumor growth or death in half of the animals (TD 50 or LD 50).

An estimate of the tumor load made on the basis of a doubling time of 18 hr in vitro (de Both et al., 1981) on day 5, yields a maximum of 9.7×10^8 RMB-1 cells. This would imply that cures can be achieved in animals containing a number of tumor cells that is approx. 3,000 times the LD 50.

A similar result was obtained by Badger and Bernstein (1983) with s.c. inoculated SL2 lymphoma cells in AKR/J mice, but in their study the therapy was started a few hours after inoculation. A cure of s.c. inoculated mice containing 500 times more tumor cells than the LD 50 was reported by Kirch and Hammerling (1981) in congeneic AKR mice. In these and most other studies allogeneic MAbs were used and similar survival was only obtained if therapy was started within 24 hr of tumor cell inoculation (Bernstein et al., 1980; Young and Hakomori, 1981; Denkers et al., 1985). In our study good results were still obtained if the therapy was delayed until the disease had progressed to between 1/4 and 1/3 of its total duration ($\pm$ 14-21 days). An initial high dose proved sufficient to activate the effector mechanisms. Good results were also reported by Kennel et al. (1983, 1985) who started treatment of virally induced sarcomas with syngeneic MAbs on the fourth day. Treatment with allogeneic antibodies may induce an anti-antibody response. This has been observed in patients treated with xenogeneic mouse MAbs, leading to reduction of therapeutic

efficacy (Miller et al., 1983) or to hypersensitivity reactions (Dillman et al., 1982). The use of syngeneic MAbs probably avoids this problem and our study shows them to be more powerful than allogeneic antibodies.

We have shown in previous studies that the MAb used for therapy binds to a tumor-restricted antigen and that, when injected in vivo, it easily reaches the tumor cells (Berends et al., 1988b). Since the present study shows efficient therapy with a high dose of IgG2a antibodies directed to a non-modulating antigen, our model may approach the limits of successful sero-immunotherapy of disseminated tumor foci.

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CHAPTER V

FACTORS INFLUENCING ANTIBODY MEDIATED CYTOTOXICITY DURING THE IMMUNOTHERAPY OF RAUSCHER VIRUS INDUCED MYELOID LEUKEMIC CELLS.

Running title: Factors involved in antibody-mediated immunotherapy

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SUMMARY

The present study was undertaken to determine the factors, which influence antibody mediated cytotoxicity during immunotherapy of virally transformed tumor cells. As model a Rauscher virus induced myeloid leukemic cell line of BALB/c origin (RMB-1) was used, which forms disseminated tumors, when inoculated intravenously in BALB/c mice. As previously reported a prolonged survival was obtained when tumor-bearing mice were treated in vivo with a single high dose of a tumor-specific IgG2a monoclonal antibody.

This study shows, that antibody dependent cellular cytotoxicity is an important mechanism involved in tumor cell destruction. Since in vitro studies showed that peritoneal macrophages were capable to kill RMB-1 cells in the presence of tumor-specific monoclonal antibody and since in the tumors of mice treated with monoclonal antibody a high influx of macrophages was observed histologically, it is likely that macrophages play an important effector role in elimination of tumor cells. Successful therapy in C5 complement deficient tumor-bearing mice suggests that complement dependent cytotoxicity does not play a major role. In nude (T cell deficient) mice the therapeutical effect of tumor-specific IgG2a antibody was significantly less than in immunocompetent mice.

Although infiltration analysis of tumors of treated and untreated mice showed equally low numbers of helper T and suppressor/cytotoxic T cells, the mortality studies of T cell deficient and immunocompetent mice indicate that T cells play a substantial, auxillary role during antibody mediated, tumor destruction in our model.

INTRODUCTION

Different opinions exist about the mechanisms, which are responsible for successful immunotherapy using monoclonal antibodies (Moabs). Although some authors favor complement dependent cytolysis (CDC) as an important factor when tumors are treated with specific Moabs (8,9), most studies emphasize the role of an antibody dependent cellular cytotoxicity (ADCC), especially for Moabs of the IgG2a subclass (1,23). Macrophages which are able to bind antibodies by Fc receptors seem the main candidates for such an ADCC in mice (26,28,39).

Uncertainty exists about the possible role of T cells in monoclonal antibody therapy. Whereas some authors assume that T cells do not play a major role (18,19), others suppose that these cells act synergistically with antibody mediated mechanisms (27).

In a previous study we used the Rauscher virus transformed myeloid leukemic cell line (RMB-1) to induce disseminated tumors by intravenous inoculation. Mice with established tumors showed prolonged or complete survival after the administration of high doses of a tumor-specific Moab of the IgG2a subclass (5).

The purpose of the present study is to investigate the role of cellular and humoral factors involved in the successful sero-immunotherapy in this model.

MATERIALS AND METHODS

Cell line

The permanently *in vitro* and *in vivo* growing R-MuLV induced myeloid cell line of BALB/c origin RMB-1 was used (11). Cells were cultured in RPMI 1640 plus 10% fetal calf serum (Seromed, Miles, Brussels) to which penicillium and streptomycin was added.

Mice

Male and female BALB/c, BALB/c nude mice and DBA/2 mice, 8-12 weeks old, were obtained from TNO, Zeist (The Netherlands) and IFFA-Credo, Lyon (France). Disseminated tumors were obtained by

intravenous (i.v.) injection of 10^7 RMB-1 cells in BALB/c mice or 5 x 10^7 RMB-1 cells in DBA/2 mice.

Monoclonal antibody

Ascites fluid containing the IgG2a monoclonal antibody IC5F5 was generated as described (4,5). This IgG2a monoclonal antibody is specific for virally induced proteins which are not expressed on normal tissues of BALB/c and DBA/2 mice. As control ascites fluid of mice bearing the non-binding hybridoma 4E3 was used.

Complement dependent cytolysis (CDC)

2 x 10^6 RMB-1 cells were incubated for 2 hours at 4°C with increasing dilutions of IC5F5 or 4E3 ascites fluid. After 45 minutes the cells were washed and fresh normal rabbit serum was added as a source of complement (1:10). Incubation was carried out for 30 minutes at 37°C. Cells were washed and the percentage of viable cells was determined microscopically in a trypan blue exclusion test. A hundred cells were counted. In every test, cells were incubated either with rabbit serum or with monoclonal antibody alone. These controls invariably contained less than 5 percent dead cells and are therefore omitted from the figure (Figure 1).

Antibody dependent cellular cytotoxicity (ADCC)

Target cell labelling

RMB-1 cells were labelled in a concentration of 10^6 cells per ml with 200 µCi $Na_2\,{}^{52}CrO_4$ (Spec. Act. 10 - 35 mCi/mmol) during 2 hours at 4°C.

For long term experiments RMB-1 cells were labelled overnight with 5 µCi ^{3}H-methyl-thymidine per ml culture fluid (Spec. Act. 5 mCi/mmol).

Effector cells

Effector cells were isolated from three different groups of mice 1. BALB/c mice i.v. inoculated with 10^7 RMB-1 cells 4 days before and treated with 0.2 ml ascites fluid containing the monoclonal antibody IC5F5 1 day before isolation. 2. idem, but treated with the non-specific monoclonal antibody 4E3 and 3. control mice without tumor and without treatment.

A. Macrophages

Macrophages were obtained by flushing the peritoneal cavity of three mice of each group with 5 ml Hanks' balanced salt solution (HBBS) and the number of nucleated cells was determined. After washing the cell suspension the cells were cultured in 96-well plates (Nunc, Denmark) during 3 hours in RPMI at 37°C. By washing the wells with culture fluid the non-adherent cells were removed and counted. The adherent macrophages formed a monolayer on the bottom of the wells. The number adherent cells were calculated by subtraction of the number of non-adherent cells from the number of cells initially present in the cell suspension.

B. Splenocytes

Splenocytes were obtained by mincing spleens of three mice, filtering the suspension through a nylon gauze and washing them thoroughly in PBS to remove cellular debris. Nucleated cells were counted in an haemocytometer.

Assay

Target cells in a concentration of 4×10^4 cells/150 µl per well were added to 10^6 effector cells per 100 µl medium (T:E ratio is 1:25). Part of the wells were treated with 10 µl IC5F5 ascites fluid whereas the other group was treated with medium only.

Short term incubation with ^{51}Cr-labelled cells was continued during 6 hours, whereas long term incubation with methyl-^3H-thymidine-labelled cells varied from 24 till 72 hours. After that period the trays were centrifuged at 800 rpm and the amount of radioactivity of 100 µl culture fluid was counted. To determine the maximum amount of radioactivity, which can be released from the target cells, wells were treated with Triton X-100 (0.1%) as lysing agent. As negative control target cells were used, which were only treated with Moabs. All experiments were carried out in quadruplicate.

$$\% \text{ specific release} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous}} \times 100$$

Therapy experiments

BALB/c and BALB/c nu/nu (nude) mice were inoculated with 10^7 RMB-1 cells and DBA/2 mice with 5×10^7 RMB-1 cells. Treatment of tumor-bearing BALB/c and nude mice was started on day 3 with a single injection of 0.2 ml IC5F5 ascites fluid. Control mice were given either no treatment or 0.2 ml non-binding ascites of hybridoma 4E3.

Tumor-bearing DBA/2 mice were daily injected with 0.2 ml IC5F5 control ascites starting 24 hours after inoculation. The ascites was incubated for 20 minutes at 56°C to inactivate complement. Mortality of the animals was monitored daily and the results were expressed graphically as percentage of survival against time. Statistical analysis was carried out as described (5).

Histology and immunohistochemistry

From a group of tumor-bearing BALB/c mice which had been treated with IC5F5 or 4E3 control ascites fluid, 2-4 mice were killed respectively 2, 4, 5, 7 and 9 days after start of treatment. Internal organs were excised, frozen in liquid nitrogen or fixed in 10% buffered formaline and embedded in paraffin. Paraffin sections were coloured with haematoxylin-azophloxin, periodic acid Schiff stain or enzymatically stained for non-specific esterase to detect macrophages. Selected sections were used for immunoperoxidase staining using Moab IC5F5 as described previously (4). Frozen sections of liver tissue were incubated with Moabs which were specific for the T cell helper subset i.e. anti-Lyt-1 and anti-L3T4 or with anti-Lyt-2 corresponding to the cytotoxic/suppressor T cell subset. Horse-radish peroxidase conjugated to rabbit anti-rat IgG (Dakopatts, Denmark; dilution 1:100) was used as a second step. Antibodies specific for macrophages could not be used because of cross-reactivity with RMB-1 cells. These Moabs were kindly provided by Drs. P.J.M. Leenen, Dept. of Cell Biology, Medical Faculty,

Rotterdam. Positive cells in and around tumor foci were microscopically scored in a semiquantitative scale from - to +++.

RESULTS

Complement Dependent Cytotoxicity

Figure 1 shows the result of a CDC assay. Over 95% of RMB-1 cells are killed after incubation with IC5F5 ascites (diluted 100 times) in the presence of rabbit complement. A rapid decline of cytotoxicity can be seen at an antibody dilution of 10^{-3} and higher (Figure 1). Without complement no cytolysis of RMB-1 cells took place in the presence of Moab.

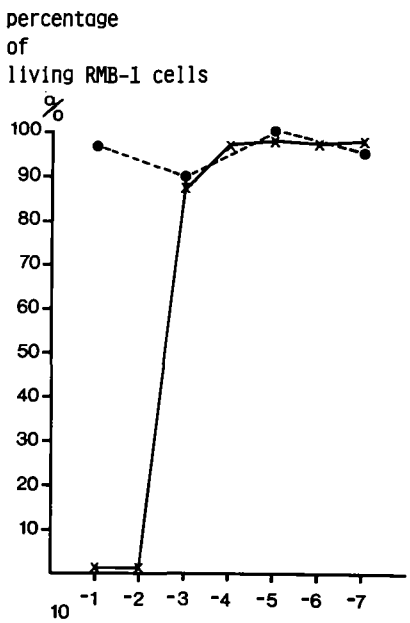

Fig. 1. Cytotoxicity of different dilutions of IC5F5 ascites (X——X) compared to control 4E3 ascites (●----●) in the presence of fresh normal rabbit serum.

Antibody dependent cellular cytotoxicity

When ^{51}Cr-labelled RMB-1 cells were incubated with peritoneal macrophages or splenocytes of the different groups of mice, the release of chromium was similar during short term incubation (till 6-8 hours), with and without antibody and did not surpass the values of control cells (data not shown). However, during long term incubations, macrophages from tumor-bearing mice were

cytotoxic to ^{3}H-methyl-thymidine-labelled RMB-1 target cells when the monoclonal IC5F5 was added. The release of ^{3}H-methyl-thymidine is comparable to the release of radioactivity of cells treated with the lysis agent Triton X-100 (0.1%). When the monoclonal antibody was omitted from the assay, lysis did not occur just as in target cells incubated with Moab alone. Macrophages of untreated tumor-bearing and control mice were also capable to kill target cells in the presence of Moab, although the lysis percentages were lower and varied between 45% and 65%. The results of one out of three experiments are presented in Figure 2. Macrophages of untreated tumor-bearing animals surprisingly had a higher cytolytic activity in the absence of Moab than macrophages of treated and control mice.

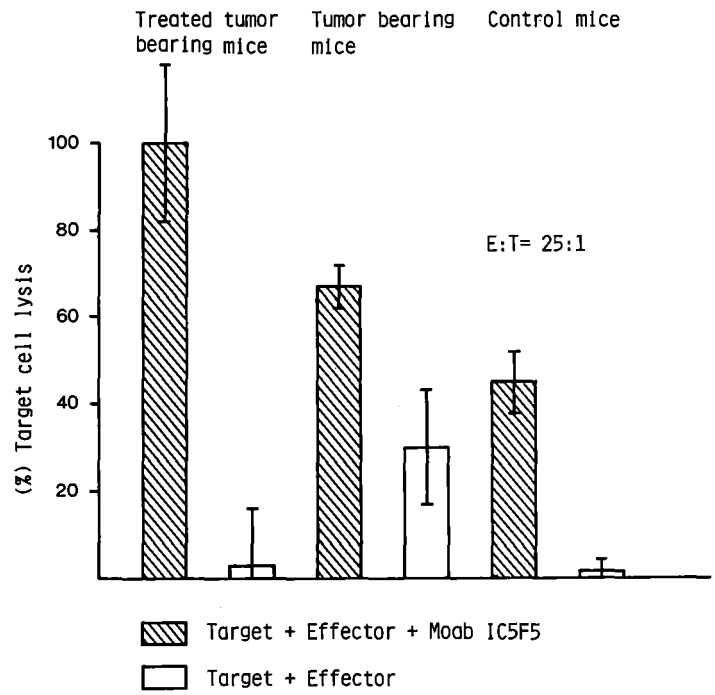

Fig. 2. Lytic activity of peritoneal macrophages as measured by ^{3}H-release, 72 hours after incubation with methyl-^{3}H-thymidine labelled RMB-1 cells. Macrophages were obtained from tumor bearing (10^{7} RMB-1 cells i.v.) mice treated with Moab IC5F5, tumor bearing non-treated and control mice. Each column represents the mean value of ± 1 SEM.

Monoclonal antibody therapy of tumor-bearing complement deficient DBA/2 mice

To assess whether CDC plays a major role we have chosen mice of the H-2 compatible DBA/2 strain as tumor-recipient. This strain is known to be C5 deficient (2). Fifty million of RMB-1 cells led within 20 days to death of the majority of mice caused by disseminated tumor-outgrowth. As shown in Figure 3 animals daily treated with 0.2 ml IC5F5 ascites starting 24 hours after inoculation of RMB-1 cells showed a considerably prolonged survival when compared to control animals ($P < 0.02$). This observation suggests that CDC does not play a major role in immunotherapy of DBA/2 mice carrying disseminated RMB-1 tumors. About 20% of tumor-bearing mice treated with 4E3 survived till 60 days after inoculation probably due to histo-incompatibility.

Monoclonal antibody therapy in T cell deficient mice

To evaluate the possible role of T cells nude mice were i.v. inoculated with 10^7 RMB-1 cells and treated 3 days later with one or repeated daily injections of IC5F5 ascites. BALB/c mice were entered in an identical scheme. In 3 repeated experiments the treated nude mice showed a significantly prolonged survival as compared to non-treated nude mice ($P < 0.01$), but the length of survival (median survival time = 22 days) was considerably less than in treated BALB/c mice (median survival time = 31 days, ($P < 0.003$). In addition it should be noted that in all experiments a proportion of BALB/c mice survived while all nude mice died. The results of one representative experiment are shown in Figure 4. These experiments indicate, that therapy with specific Moab is effective and that T cells play an auxillary role in successful immunotherapy with Moabs.

Infiltration analysis of treated and untreated mice

Mice, which were treated with daily injections of IC5F5 or 4E3 ascites from the fifth day after i.v. inoculation, were killed on day 2, 4, 5, 7 and 9 after start of therapy and organs were histologically analysed. In paraffin sections of liver tissue of treated mice a striking influx of macrophages was seen in liver tumor foci of treated animals from the fourth day after onset of therapy (Figure 5a). After 4 days of therapy, differen-

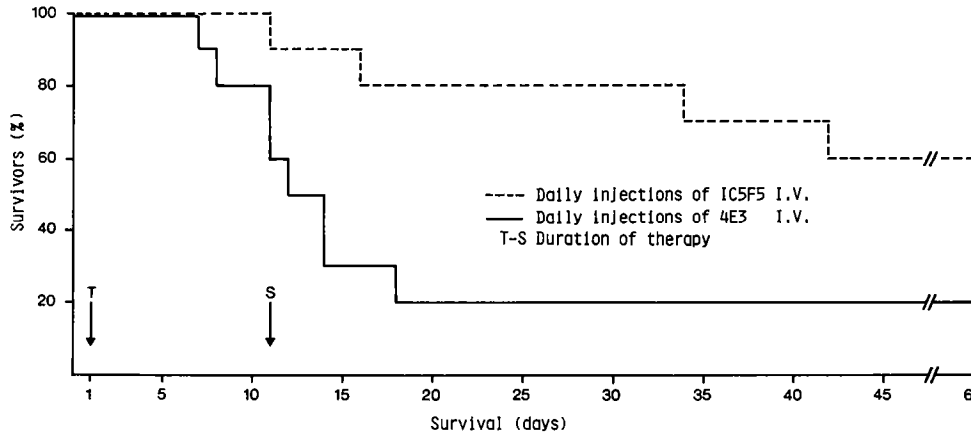

Fig. 3. Survival of complement factor C5 deficient DBA/2 inoculated i.v. with 5 x 10^7 RMB-1 cells 24 hours before treatment with 0.2 ml IC5F5 ascites or 4E3 ascites as control. n = 10 for each group.

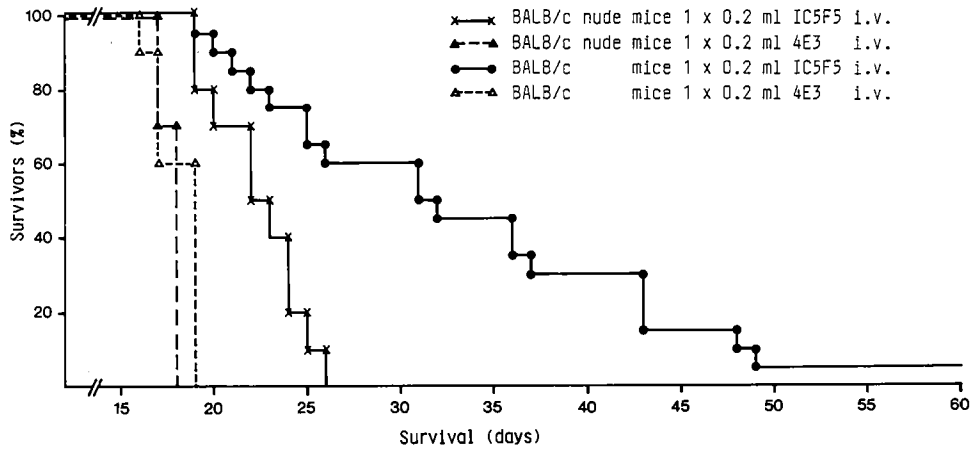

Fig. 4. Survival of BALB/c and BALB/c nude mice inoculated i.v. with 10^7 with RMB-1 cells 72 hours before treatment with one single injection of 0.2 ml IC5F5 ascites or 4E3 ascites as control. BALB/c: n = 20 for each group; BALB/c nude mice: n = 10 for each group.

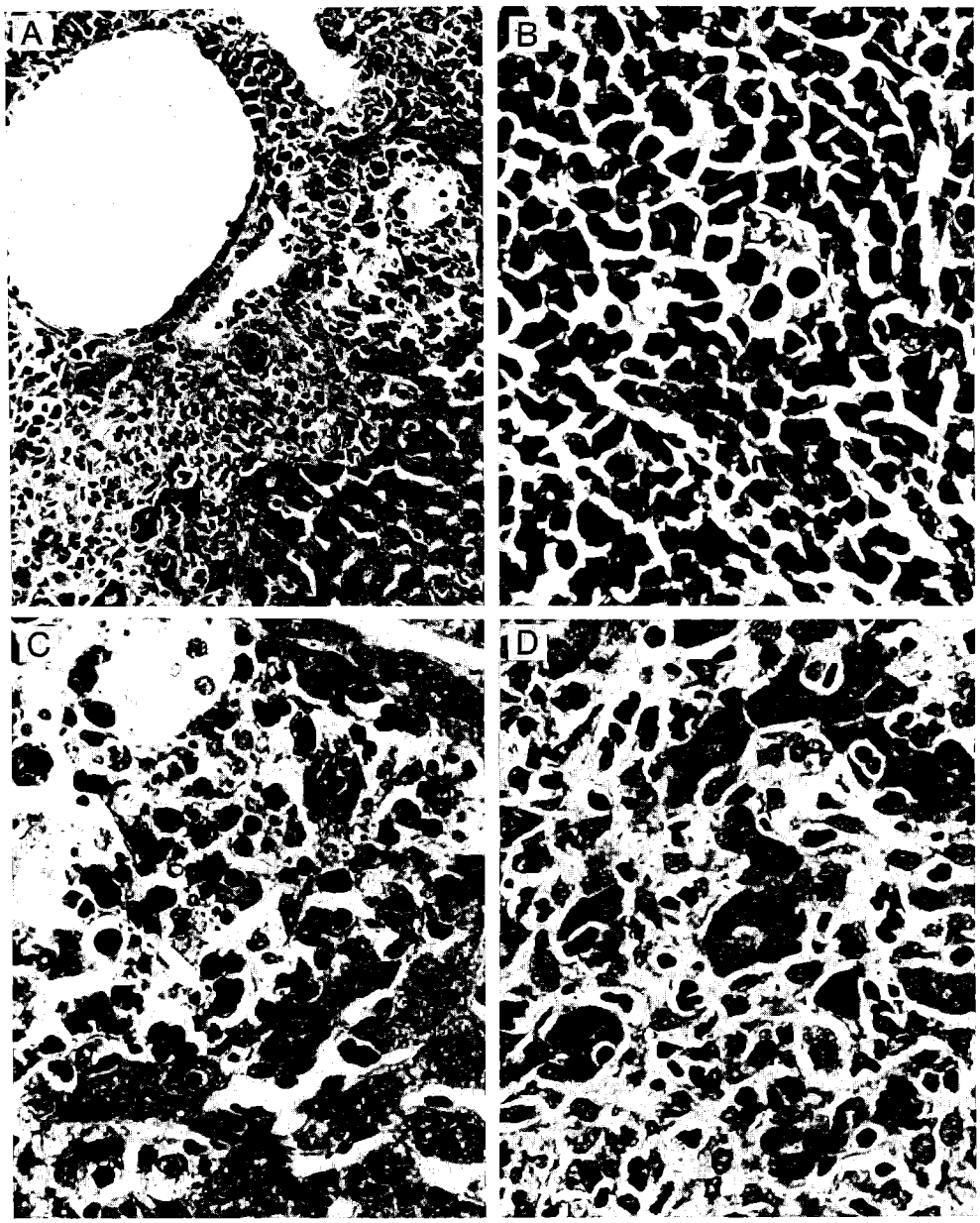

Fig. 5.
a) RMB-1 tumor in the liver on the fourth day after treatment with 0.2 ml IC5F5 ascites;
b) idem; edema around tumor cells;
c) idem; macrophages with phagocitized dead RMB-1 cells;
d) idem; fibroblastic proliferation after tumor destruction.

ces in tumor-extension versus destruction between the treated and non-treated mice were most evident. In IC5F5 treated mice edema was observed between tumor cells (Figure 5b) and phagocytizing macrophages were often seen in large areas of destructed tumor tissue (Figure 5c). Only bile ducts and some distorted foci of hepatocytes were left intact. The lesions showed repair tissue with extensive fibroblastic proliferation and deposition of collagen (Figure 5d). In 4E3 treated control mice tumor foci rapidly increased in size within a few days and scattered macrophages were seen in considerable lesser numbers as compared to IC5F5 treated mice. Both in treated and in control mice some infiltrates contained polymorphonuclear leucocytes, but no major influx of lymphocytes could be detected.

Involvement of T-lymphocytes in treated and untreated tumor-bearing mice was also investigated by testing cryostate sections with the monoclonal antibodies anti-Lyt-1+, anti-L3T4 (helper T cell) and anti-Lyt-2+ (cytotoxic/suppressor T cells). Unfortunately, the common leukocyte antigen marker T200 and common T cell marker Thy-1 could not be used because of cross-reactivity with the RMB-1 tumor cells (12). Using anti-Lyt-1+ and -Lyt-2+ antibodies, only few positive cells were found in tumor foci in the livers of control animals. A slightly larger number of Lyt-1+ and Lyt-2+ positive cells was sometimes present in IC5F5 treated BALB/c mice. Lyt-1+ and Lyt-2+ positive cells occurred at nearly equal numbers at all timepoints investigated (Figure 6a; 6b) and no changes in relative number of Lyt-1+ or Lyt-2+ positive cells were observed at the various time intervals after start of treatment.

An increase in number of cells positive for the helper T marker L3T4 could not be established in treated mice as compared to untreated tumor-bearing mice. It can be concluded from these histological observations that in particular macrophages seemed to be the effector cells responsible for tumor cell destruction.

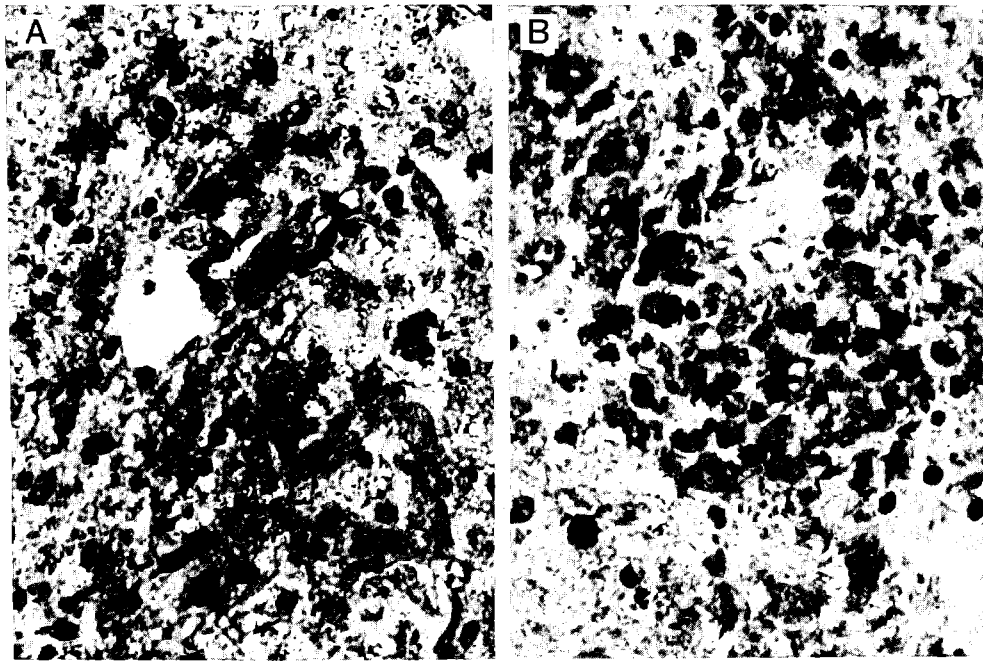

Fig. 6. Immunoperoxidase staining of frozen sections of liver with RMB-1 tumors 4 days after treatment with IC5F5 ascites:
a) Incubation with anti-L3T4
b) incubation with anti-Lyt2+

DISCUSSION

Several alternative mechanisms have been reported, which may account for successful sero-immunotherapy. The potential of direct *in vitro* growth inhibition has been attributed to monoclonal antibodies, which can block receptor functions. For instance, the receptor for the viral envelope protein on lymphoma cells could be blocked by an antibody against gp70 preventing that gp70 acts as mitogen (34).

Similarly, both *in vitro* and *in vivo* growth inhibition is reported by Masui et al. (33) using Moabs against the epidermal growth factor receptor on a carcinoma cell line, and by Sauvage et al. (37) using Moabs blocking the transferrin receptor of a leukemic cell line. A direct anti-proliferative effect on

B cell neoplasms by anti-idiotypic Moabs has been described also (31).

A more subtle approach is the generation of anti-idiotype Moabs. Such anti-idiotype antibodies may interact with T cells, which express the corresponding idiotype and which are part of the putative idiotype network regulating the anti-tumor immune response. Modulation of such a network by the use of anti-idiotype antibodies, may lead to tumor-destruction by a delayed-type hypersensitivity reaction (35).

Until now, immunotherapy models are based on the application of Moabs directed against viral antigens on tumors (21,27,38), differentiation antigens (3,6,27) or tumor-associated antigens on xenografts (18,19). In these cases direct growth inhibition of tumor cells by antibody-antigen interaction was not reported, but instead the Moabs appeared to mediate cytotoxicity. It is thought that tumor-cytotoxicity in most of these models is caused by an ADCC, probably mediated by activated macrophages (1,20). This is especially true for immunotherapy with antibodies of the IgG2a subclass. In our study a Moab of the IgG2a isotype is used which evokes an ADCC to RMB-1 tumor cells by macrophages, while in in vitro studies this antibody was also fully capable of CDC in the presence of rabbit complement.

When DBA/2 mice are inoculated with high doses of RMB-1 cells, which are of BALB/c origin, almost all mice died of disseminated tumor. Apparently the minor histocompatibility differences between BALB/c and DBA/2 mice are not sufficient to prevent tumor cell outgrowth in most mice. As in these C5 deficient DBA/2 mice immunotherapy was successful, it may be concluded that complement dependent cytotoxicity is not a major effector mechanism in this model. This is in accordance with other studies in AKR mice, which are also C5 deficient (3,29). An additional argument against the involvement of CDC is the observed delayed tumoricidal effect in vivo after the injection of Moabs.

Histological studies revealed the abundant presence of macrophages as also observed by others during immunotherapy (1) or during spontaneous tumor-regression (22). We did not observe

extensive phagocytosis of morphologically viable cells, but many macrophages were found surrounding or phagocytizing dead cells. The long latency phase of about 4 days after therapy, before macrophage activation was morphologically at its height point, may reflect a slow ADCC mechanism which reaches its maximum after 48-72 hours (1,20). Our in vitro studies confirmed the capability of the IgG2a antibody IC5F5 to elicit a slow ADCC to RMB-1 cells. Strikingly in vitro some lytic activity was seen with macrophages attained from untreated tumor-bearing mice when no antibody in the ADCC test was applied. This phenomenon was not present when macrophages were used from treated tumor-bearing mice. Macrophages from these latter mice were only capable of slow ADCC. Possibly some effector cells of untreated tumor-bearing mice are activated for direct antibody-independent lysis, whereas the majority of those of treated mice is mainly primed for slow ADCC. These findings correlate well with the results of Johnson et al. (20) who proved macrophages to be capable of mediating either direct lysis or slow ADCC but not both.

A diminished immunotherapeutical response of nude mice inoculated with Moloney sarcoma virus transformed cells to i.v. treatment was also found by Kennel et al. (21) and Lamon et al. (27). These and our results suggest an additional role of T cells. This hypothesis is corroborated by the presence of both helper T cells (Lyt-1+ and L3T4+) and supressor/cytotoxic T cells (Lyt-2+) cells in infiltrates surrounding tumor cells (Figure 6).

Cytotoxic Lyt-2+ T cells are able to destruct cells which express viral antigens. In retrovirus infected cells such a mechanism has repeatedly been reported (16,17,30), but today doubt exists if Lyt-2+ cells are the only T cell subset involved in cellular mediated cytotoxicity (15,36). Since the action of Lyt-2+ cytotoxic T cells is known to be independent of antibodies and since histologically only small numbers of Lyt-2+ cells are present, it is not probable that cytotoxic T lymphocytes play an important role in our model. Moreover, their numbers are hardly different from those in untreated mice. Similar observations were made in rats with progressing and regressing tumors (32).

Evidence for a role of helper T cells in immunotherapy comes also from the cellular transfer of IL-2 stimulated helper T cells, which are able to induce regression of Friend and Rauscher virus induced tumors (7,10). It is likely that sensitized helper T cells produce macrophage activating lymphokines which promote the influx of macrophages (13,14). This process shows similarities to delayed-type hypersensitivity, a type of immune response which can indeed be induced by injection of irradiated RMB-1 cells in BALB/c mice (25). Therefore we hypothesized that in our immunotherapy model T cell mediated influx of macrophages around tumor foci may lead to a more effective ADCC and which ultimately in some mice may result into complete eradication of tumor cells.

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CHAPTER VI

ENHANCEMENT AND SUPPRESSION OF DTH REACTIVITY TO RAUSCHER MURINE LEUKEMIA VIRUS INDUCED TUMOR CELL LINES

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SUMMARY

Delayed-type hypersensitivity (DTH) to Rauscher Murine Leukemia Virus (R-MuLV)-encoded or -induced determinants was induced in mice by three syngeneic R-MuLV-induced tumor cell lines, i.e. a myeloid tumor, RMB-1, an erythroid tumor, RED-1 and a lymphoid tumor, RLD-1.

DTH to subcutaneously (s.c.) administered RMB-1 cells appeared on day 4, with a maximum DTH response on day 6 or 7. The induction of DTH could be prevented by a tolerizing intravenous (i.v.) injection of R-MuLV-induced tumor cells several days before the s.c. immunization.

The three R-MuLV-induced tumor cell lines showed cross reactivity in the DTH assay, whereas no cross reactivity was found with syngeneic WEHI-3 cells. This indicates that the three R-MuLV-induced tumor cell lines share a virally encoded or induced antigenic determinant, which activates T cells.

When the RMB-1 cells used for immunization had been cultured in medium supplemented with interferon- γ (IFN-γ), the subsequent DTH response was increased. This coincided with an increased expression of the R-MuLV-specific antigenic determinants on RMB-1 cells as demonstrated by Scatchard analysis.

INTRODUCTION

Immunological tumor-rejection depends on the presence of antigenic determinants on the tumor cells, which are not usually present on their normal counterparts. Such antigenic determinants may be induced by chemical, viral or physical agents and can also be found on spontaneously arising tumors (Halliday and Webb, 1969; Morton et al., 1969; Kripke, 1981; Galetto et al., 1985). Cells transformed by RNA tumor viruses express virally encoded proteins, and so called virus associated proteins (Nowinski et al.,1978; Rogers et al., 1984). These neoantigens can induce antibody formation as well as cellular immune responses, such as cellular cytotoxicity and delayed-type hypersensitivity (DTH) (Levy and Leclerc, 1977).

DTH responses can be easily elicited to various antigens, such as bacteria, viruses, xenogeneic red blood cells, and contact sensitizing agents (reviewed by Crowle, 1975). Also tumor cells can induce DTH (Halliday and Webb, 1969; Hawrylko, 1980; Hoover et al., 1984). DTH reactions are mediated by T cells, particularly by the L3T4 positive helper T cell subset (Mossman and Coffman, 1987). Evidence is increasing that Lyt-1+2-, L3T4+ cells, depending on the experimental conditions, can also mediate tumor rejection (Ozawa et al., 1986; Paul et al., 1987; Bookman et al., 1987).

Several adjuvants have been used to increase the anti-tumor response, such as BCG (Hawrylko, 1980) and Corynebacterium parvum (Dye et al., 1981). One could also increase the anti-tumor response by enhancing the immunogenicity of the tumor for instance by using haptenated tumor cells (Suda et al., 1986) or by treatment with interferon, which is known to increase the expression of MHC-coded antigens (Tanaka et al., 1986).

In previous studies we reported about two R-MuLV-specific monoclonal antibodies (MAbs) raised against a R-MuLV-induced myeloid tumor (Berends et al., 1988 a-c). One of the MAbs, which recognizes virally encoded proteins on RMB-1 cells, was successfully used in immunotherapy. A striking difference was found between the effect of this MAb therapy in T cell deprived nude

mice and their euthymic littermates. This led us to suggest, that a T cell dependent anti-tumor immune response is involved. In the present study we therefore investigated the ability of BALB/c mice to give rise to a DTH response after s.c. immunization with R-MuLV-induced tumor cells.

After having established that R-MuLV-induced tumor cells indeed evoke DTH, we investigated the specificity of this response, its suppression by a tolerizing i.v. preimmunization with irradiated tumor cells and the effect of interferon-γ (IFN-γ) on the expression of the R-MuLV-induced tumor cell surface antigens and on the immunogenicity of RMB-1 cells as measured in the DTH assay.

MATERIALS AND METHODS

Mice

BALB/c (H-2^d) and DBA/2 (H-2^d) female mice, four weeks of age, were purchased from Bomholtgard, Ry, Denmark. BALB/c (H-2^d) female mice, four weeks of age, were purchased from HARLAN OLAC Ltd, Bicester, U.K. Other BALB/c (H-2^d) female and male mice were bred at the Dept. of Cell Biology, Immunology and Genetics of the Erasmus University.

Cell lines

Three R-MuLV-induced tumor cell lines were used: a myeloid tumor cell line of BALB/c origin (RMB-1), a lymphoid tumor cell line of DBA/2 origin (RLD-1), and an erythroid tumor cell line of DBA/2 origin (RED-1) (De Both et al., 1978; 1981; 1983; 1985). The WEHI-3 immature macrophage cell line of BALB/c origin, originally described by Warner et al. (1969), was used as a control. This cell line does not express R-MuLV antigens. All cell lines were cultured in RPMI 1640 tissue culture medium, supplemented with 10% fetal calf serum, glutamin (4 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and if necessary 100-150 U IFN-γ/ml, in a humidified atmosphere with 5% CO2. IFN-γ was supplied by Drs. M. van Heuvel and I.J. Bosveld as culture supernatant from a chinese hamster cell line with the

amplified murine recombinant IFN-γ gene (Dijkmans et al., 1985) containing 3 x 10^4 IU/ml. As a control culture supernatant from the CHO12RO cell line was used (Stefanini et al., 1982).

Antigen density on RMB-1 cells

To quantitate the density of a R-MuLV-encoded surface antigen of RMB-1 cells, a Scatchard analysis was performed, using the specific MAb IC5F5 that was previously described (Berends et al., 1988a). Briefly, RMB-1 cells were incubated for 90 minutes at room temperature in a volume of 100 µl of Hanks' Balanced Salt Solution (HBSS), supplemented with 20 mM HEPES and 0.125% gelatin containing varying amounts (0.01-10 nM) of ^{125}I-IC5F5. Iodination of the MAbs was earlier described (Berends et al. 1988b). For the determination of non-specifically bound ^{125}I-IC5F5, the cells were incubated with varying amounts of ^{125}I-IC5F5 in the presence of excess (10^{-6} M) unlabelled IC5F5 for 90 min. Thereafter the cells were washed to remove unbound IC5F5. Cell-bound radioactivity was determined in an LKB 1280 ultra-gammacounter. The association constant (Ka) and the number of binding sites were calculated according to Scatchard (1949).

Induction of DTH reactivity

DTH was induced by s.c. immunization of the mice with 3 x 10^7 irradiated (20 Gy) tumor cells suspended in a volume of 300 µl. A volume of 150 µl of this suspension was injected into each inguinal area.

Assay for DTH

DTH responses were elicited by s.c. injection of 3 to 6 x 10^6 irradiated (20 Gy) tumor cells, suspended in a volume of 50 µl, into the dorsum of the right hind foot seven days after the induction of DTH. Unimmunized control mice received this challenge only. The difference in thickness of the right and left hind foot was measured 24 hours later. The specific DTH response was calculated as the relative increase in foot thickness of the immunized mice minus the relative increase in foot thickness of

the control mice. The increase of foot thickness of the control mice ranged between 10 and 20%.

Induction of suppression

Suppression of DTH was induced by i.v. administration of a high dose of heavily irradiated (80 Gy) tumor cells, seven days prior to the induction of DTH. The administered number of cells is indicated in the legends to the figures.

RESULTS

Induction of DTH reactivity to R-MuLV-induced tumor cells

BALB/c responder mice were s.c. immunized with varying doses of syngeneic RMB-1 cells. A maximal DTH response was found after s.c. immunization with 3 x 10^7 RMB-1 cells (data not shown). This dose was used during all the experiments described. DBA/2 responder mice were s.c. immunized with 3 x 10^7 syngeneic RED-1 or RLD-1 tumor cells. Seven days after s.c. immunization all mice were challenged with 6 x 10^6 similar tumor cells as used for immunization.

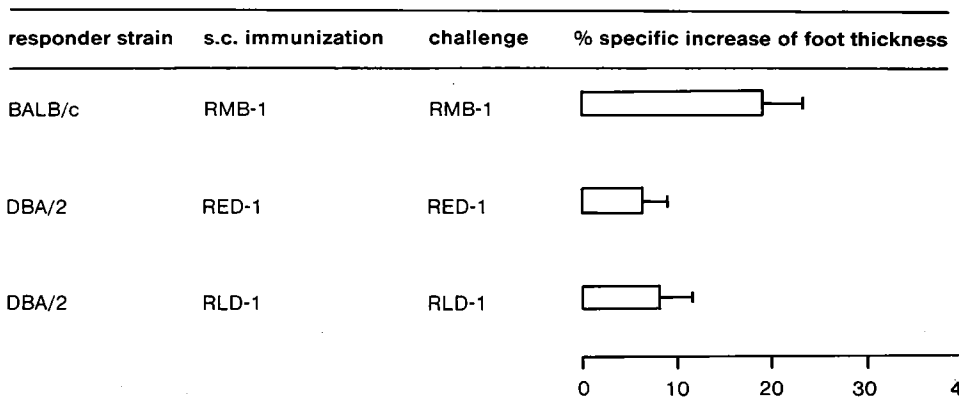

Fig. 1. DTH reactivity to RMB-1, RED-1 and RLD-1 tumor cells. BALB/c mice were s.c. immunized with 3 x 10^7 RMB-1 cells and challenged for DTH with 6 x 10^6 RMB-1 cells 7 days later. DBA/2 responder mice were s.c. immunized with 3 x 10^7 RED-1 or RLD-1 cells and challenged for DTH 7 days later with 6 x 10^6 RED-1 and RLD-1 cells, respectively. Each column represents the mean response ± 1 SEM (n = 5).

The RMB-1 cell line induced a pronounced DTH response, whereas the responses induced by RED-1 and RLD-1 were weak (Fig. 1).

Kinetics of the DTH response to RMB-1 cells

Several groups of BALB/c responder mice were s.c. immunized with 3 x 10^7 RMB-1 cells. At various days after immunization, individual groups were challenged with 3 x 10^6 RMB-1 cells. From day 4 after immunization DTH was detectable with a maximum response around day 7 (Fig. 2).

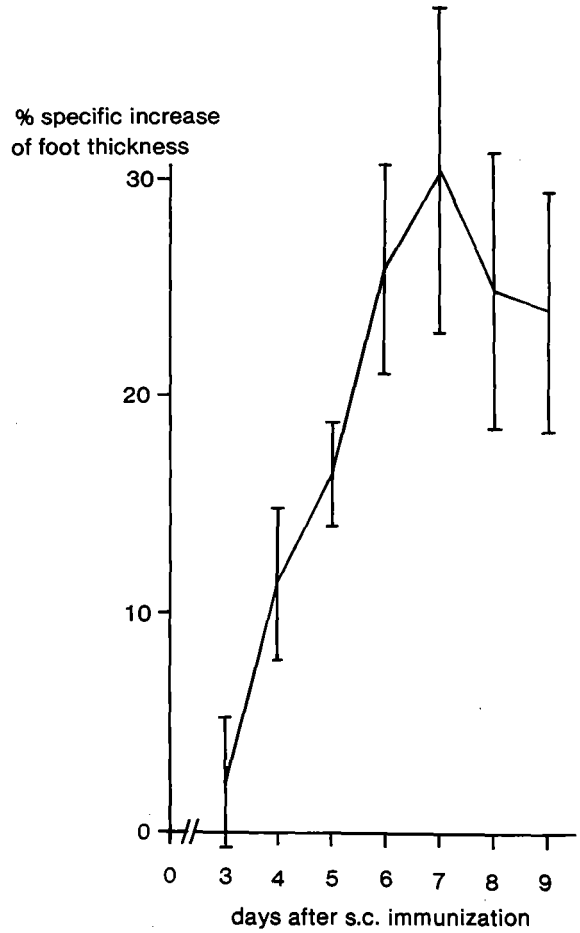

Fig. 2. Kinetics of the DTH response to RMB-1 cells. BALB/c mice were s.c. immunized with 3 x 10^7 RMB-1 cells. DTH reactivity was determined at various intervals after s.c. immunization. Each experimental point represents the mean repsonse ± 1 SEM (n = 5).

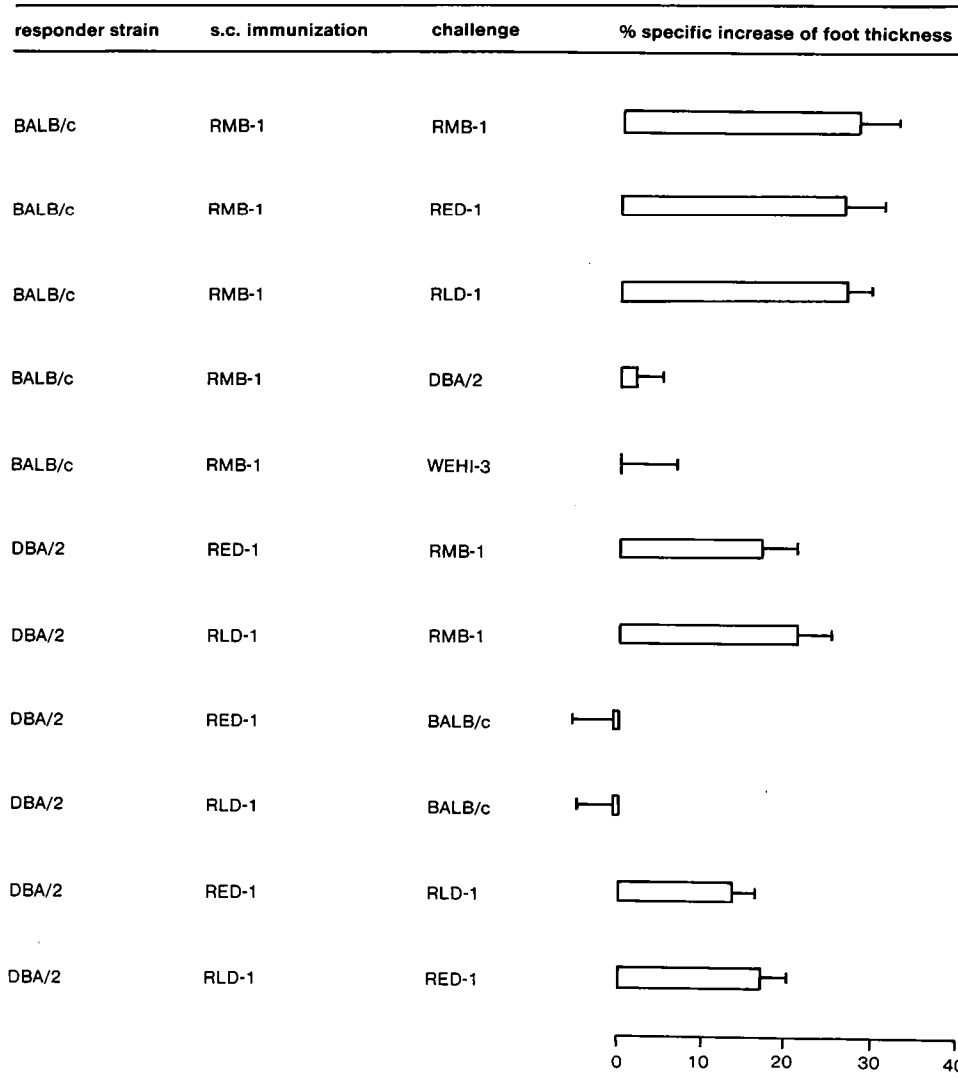

Fig. 3. Cross-reactivity of tumor associated antigens on RMB-1, RED-1 and RLD-1 cells. Several groups of BALB/c mice were s.c. immunized with 3 x 10^7 RMB-1 cells and challenged for DTH with 6 x 10^6 RMB-1, RED-1 or RLD-1 cells. Control mice were challenged with 6 x 10^6 DBA/2 spleen cells or WEHI-3 cells. DBA/2 responder mice were s.c. immunized with 3 x 10^7 RED-1 or RLD-1 cells and challenged for DTH with 6 x 10^6 cells. A DBA/2 control group was challenged with 6 x 10^6 BALB/c spleen cells. All cell lines had been cultured with IFN-γ for the last 24 hours. Each column represents the mean response ± 1 SEM (n = 5).

Cross reactivity between tumor-associated antigens on different R-MuLV-induced cell lines

To investigate whether the DTH reactive T cells responding to the R-MuLV-induced tumor cell lines recognize a common (presumably viral) determinant we investigated the cross reactivity in the DTH assay. Thus, BALB/c mice were s.c. immunized with 3×10^7 RMB-1 cells and challenged for DTH seven days later with 6×10^6 RMB-1, RED-1 or RLD-1 cells. As a control groups of BALB/c mice were challenged with 6×10^6 DBA/2 spleen cells or with WEHI-3 cells. Fig. 3 shows that after immunization with RMB-1 cells challenge with each of the three tumor cell lines led to a substantial DTH response (lines 1-3). Challenge with DBA/2 spleen cells or with the WEHI-3 cell line, however, did not cause a DTH response (lines 4 and 5). Cross reactivity between the three R-MuLV-induced tumor cell lines was also found after s.c. immunization of DBA/2 responder mice with 3×10^7 RED-1 or RLD-1 cells and challenge with RMB-1 cells (lines 6 and 7), whereas no DTH was found after challenge with BALB/c spleen cells (lines 8 and 9). Cross reactivity was also determined after s.c. immunization with RED-1 cells and challenge with RLD-1 cells and vice versa (lines 10 and 11).

Suppression of DTH to RMB-1 cells

After i.v. injection of BALB/c responder mice with either 1×10^7 or 5×10^7 irradiated RMB-1 tumor cells, s.c. immunization with 3×10^7 RMB-1 tumor cells did no longer induce a state of DTH (Fig. 4). Previous studies have shown that such an i.v. preimmunization induced suppressor T (Ts) cells, which can suppress the subsequent induction or elicitation of DTH (Van der Kwast et al., 1981, Bianchi et al., 1984). This suppression was found independent of whether or not the RMB-1 cells had been treated with IFN-γ.

Effects of IFN-γ on antigen-expression and DTH reactivity

IFN-γ is known for its enhancing effect on the expression of various antigens. We studied the effect of IFN-γ on the expression of R-MuLV-induced antigens. To this end IFN-γ was added to the culture medium of RMB-1 cells for 24 or 48 hours. Stimulation

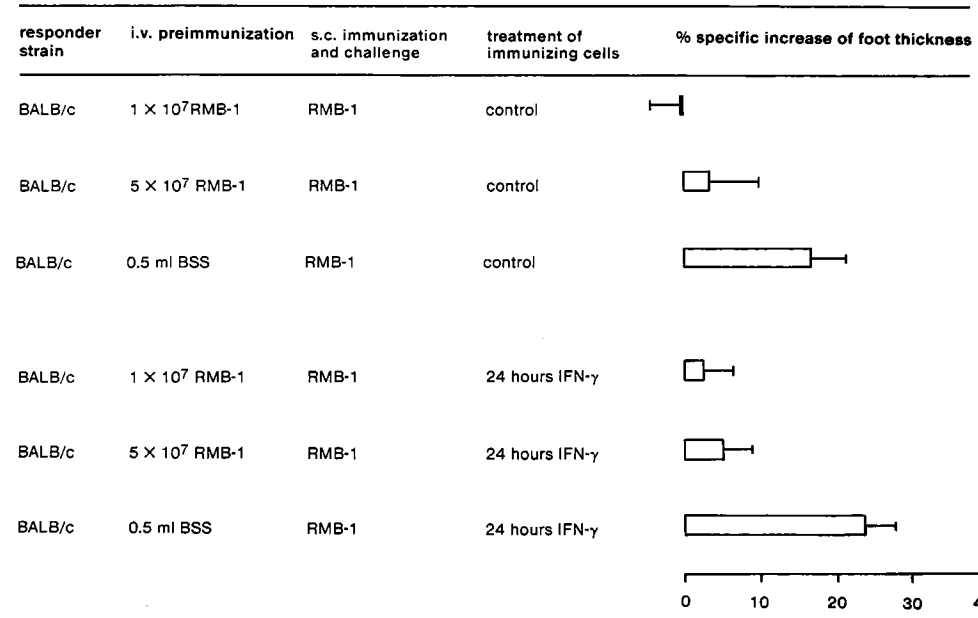

Fig. 4. Induction of a state of suppression by i.v. administration of RMB-1 cells. BALB/c mice were i.v. pre-immunized with either 1 or 5 x 10^7 RMB-1 cells. In one experiment the RMB-1 cells used had been cultured in IFN-γ for 24 hours, in the other untreated mice were s.c. immunized with 3 x 10^7 RMB-1 cells. Another 7 days later the mice were challenged with 6 x 10^6 RMB-1 cells. Each column represent the mean reponse ± 1 SEM (n = 5).

of RMB-1 cells with IFN-γ for 24 hours caused a nearly twofold increase in the amount of binding sites on RMB-1 cells detected by the binding of ^{125}I-labelled-IC5F5 (Fig. 5A), and a slight decrease in affinity (Fig. 5B). This coincided with a strong enhancement of DTH (Fig. 6). After culture of RMB-1 cells with IFN-γ for 48 hours the increase in R-MuLV antigen-expression was much smaller than in RMB-1 cells that had been cultured with IFN-γ for 24 hours. In this case also no enhancing effect in the DTH assay was found. Apparently the IFN-γ -induced increased expression of R-MuLV-encoded proteins by RMB-1 cells is only temporary. This increased antigen-expression was not due to an increase in the cell size (data not shown).

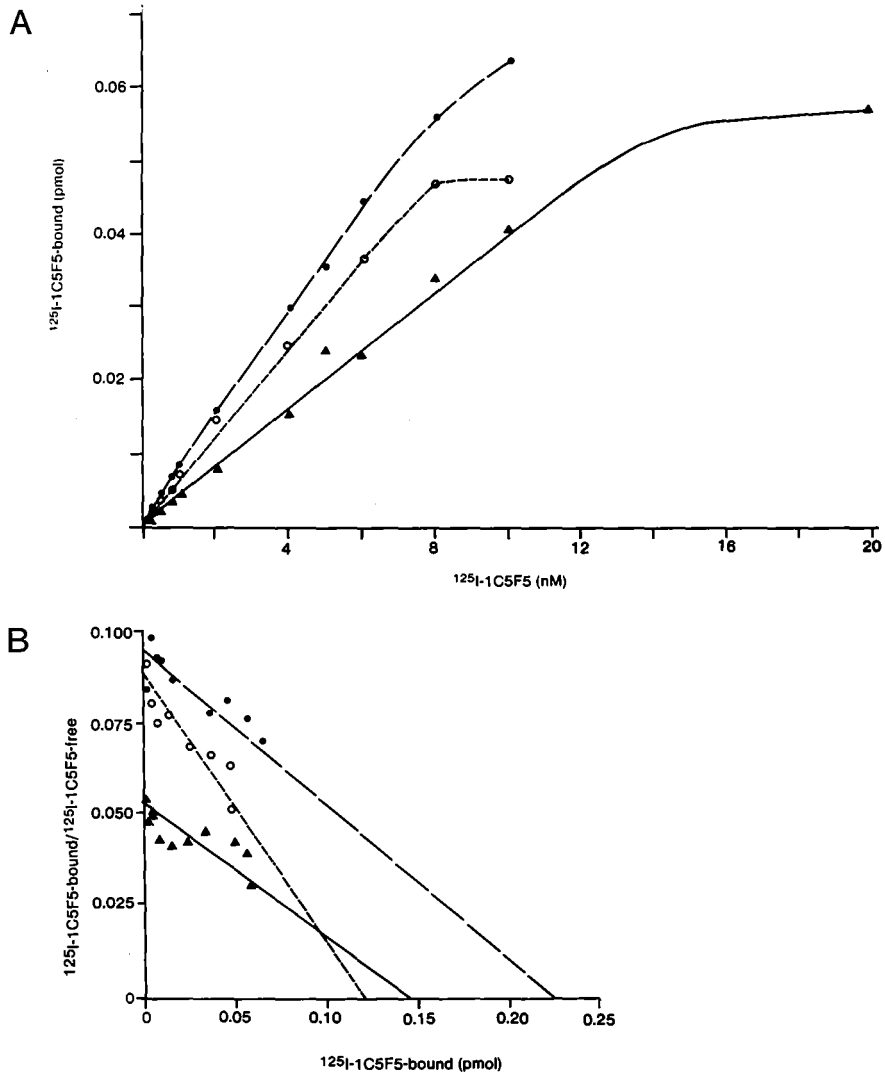

Fig. 5. Determination of the number of binding sites and the association constant (Ka) of ^{125}I-labelled IC5F5 MAbs on RMB-1 cells by Scatchard analysis. (A) a constant number (4×10^5) of RMB-1 cells was incubated with varying amounts (0.01-10 nM) of ^{125}I-IC5F5. For the determination of non-specifically bound ^{125}I-IC5F5, the cells were incubated with excess (10^{-6} M) unlabelled IC5F5 for 90 minutes. B max is the number of binding sites under saturated conditions. (B) Ka was calculated by the quotient of ^{125}I-IC5F5-bound/^{125}I-IC5F5-free as compared to the amount of ^{125}I-IC5F5-bound.
(O) untreated RMB-1 cells; B max: 180,000/cell; Ka: 7.4×10^8 l/mol
(●) RMB-1 cells cultured with IFN-γ for 24 hours; B max: 335,000/cell; Ka: $4,2 \times 10^8$ l/mol
(▲) RMB-1 cells cultured with IFN-γ for 48 hours; B max: 218,000/cell; Ka: $3,6 \times 10^8$ l/mol.

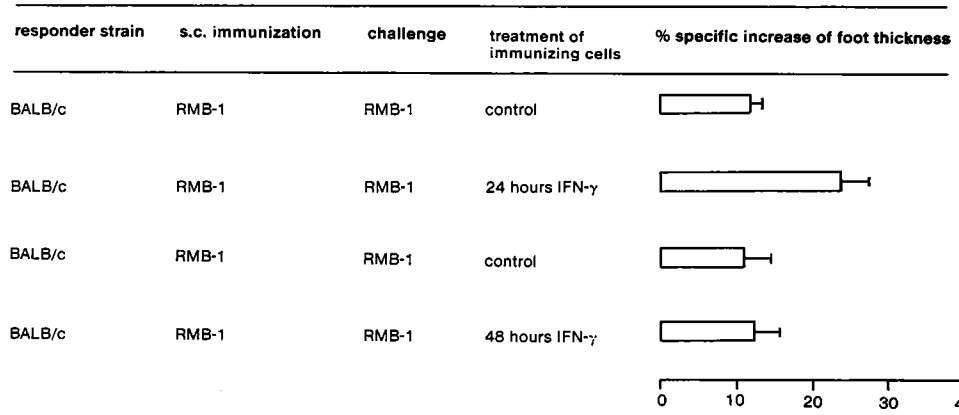

Fig. 6. Effect of IFN-γ on the immunogenicity of RMB-1 cells. BALB/c mice were s.c. immunized with 3 x 10^7 RMB-1 cells that had been cultured with IFN-γ for 24 or 48 hours. As a control mice were immunized with RMB-1 cells that had been cultured in the absence of IFN-γ. Seven days later all mice were challenged for DTH with 6 x 10^6 similar RMB-1 cells. Each column represents the mean response ± 1 SEM (n =5).

DISCUSSION

This study shows that R-MuLV-induced tumor cell lines induce DTH in syngeneic mice. This T cell dependent response is more pronounced in the case of RMB-1 cells, than in the case of RED-1 and RLD-1 cells. This correlates with the fact that the latter two cell lines differ from the RMB-1 cell line in that they are virus-producing, whereas RMB-1 is not. Moreover, RMB-1 cells grow s.c. only after inoculation of high numbers of cells whereas RED-1 and RLD-1 cells do even after s.c. inoculation of low numbers (unpublished results).

The maximum DTH response to RMB-1 cells was found seven days after s.c. immunization. This is in accordance with reports by others (Hawrylko et al., 1980). DTH responses to histocompatibility antigens generally peak one or two days earlier.

All three different R-MuLV-induced cell lines could induce DTH although not equally strong. We investigated whether DTH induced by s.c. immunization with one cell line could be elicited

by challenge with another R-MuLV-induced cell line. Cross reactivity was found in all combinations tested. The response was the highest when RMB-1 cells were used for immunization or challenge. The cross reactivity between RED-1 and RLD-1 cells was relatively weak, as could be expected in view of the weak DTH responses they evoke. Together the results suggest that the DTH response is directed to a common R-MuLV-encoded or -induced tumor-associated antigen, most clearly expressed on RMB-1 cells.

Even if tumors possess immunogenic determinants they can escape elimination by the immune system. The mechanisms involved could be the release of suppressive factors by the tumor cells (Mizel et al., 1980), or the induction of suppressor cells (for a review see North, 1985). Here we report the induction of suppression of the subsequent DTH response, by administration of a high dose of heavily irradiated tumor cells. Most probably the huge amount of tumor antigens leads to the induction of the state of suppression. Previously we investigated extensively the mechanism of suppression of the DTH response to H-2 and non-H-2 histocompatibility antigens after i.v. preimmunization, which proved to be due to the induction of antigen-specific Ts cells (Van der Kwast et al., 1981; Bianchi et al., 1984). Despite of this, the possibility should be taken into account that factors released by the (irradiated) tumor cells can also give rise to the immunosuppression. Particularly the retroviral protein p15E should be mentioned in this respect (Bendinelli et al., 1985).

When suppression can be induced by a huge amount of tumor antigens presented to the immune system, this phenomenon might play a role in clinical practice, since frequently irradiation is used in the therapy of malignant tumors, which leads to massive tumor reduction, and therefore to the appearance of large amounts of tumor-derived antigens in the bloodstream. This in turn could lead to suppression of the induction of an adequate immune response.

One approach to achieve tumor rejection is to prevent or to block a possible immunosuppressive mechanism. Alternatively one might enhance the hosts' anti-tumor response using adjuvants, such as BCG (Hawrylko, 1980) and Corynebacterium parvum (Dye et

al., 1981). A third way would be the enhancement of tumor immunogenicity. Suda et al. (1986) reported increased anti-tumor immunity using haptenated tumor cells.

Here we show that IFN-γ can temporarily enhance the expression of tumor-specific antigens. Interestingly, in parallel, the in vivo anti-tumor DTH response was enhanced to the same extent (Fig. 1). Various authors asked attention for the immunomodulation of interferons (for reviews see Priestman, 1979; Krim, 1980). Interferons should exert their effects via at least three mechanisms e.g. direct cytotoxicity, increased MHC-antigen expression on the tumor cells and increased host-mediated anti-tumor effects. Most studies on the effect of interferons on tumor immunogenicity focus on the increase of the expression of MHC antigens on the tumor cells (King and Jones, 1983; Green and Phillips, 1986), which would lead to improved recognition by the hosts' immune system and subsequently may result in better tumor-rejection. Accumulation of immature viral budding particles at the cell surface due to inhibitory action of IFN α/β has been described before (Friedman et al., 1980). The present study shows that IFN-γ can also enhance the expression of a virally encoded or induced tumor antigen. Greiner et al. (1987) demonstrated the increased expression of a tumor-associated antigen on a human colon-xenograft after treatment with IFN-α. Such phenomena are especially of interest when a tumor specific MAb is available. Possibly, the effectiveness of MAb therapy of tumors can be improved by simultaneous treatment with a tumor antigen-enhancing lymphokine as IFN-γ. Our results suggest that interferons can also be important in the enhancement of the cellular anti-tumor immune response.

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CHAPTER VII

GENERAL DISCUSSION

This thesis deals with the effect of syngeneic monoclonal antibodies on tumor elimination.

The model described in the foregoing chapters consists of a Rauscher virus induced myeloid leukemic cell line (RMB-1), the cells of which form disseminated foci after intravenous inoculation. Although the leukemia from which the line originates is virally induced and the cells express viral proteins, the RMB-1 cells do not produce virus.

When inoculated in BALB/c mice an immune response in the host is evoked, which makes the generation of monoclonal antibodies possible. One of these monoclonal antibodies was selected and proved to be directed to gag-related viral proteins, which are consistently expressed on RMB-1 cells (Chapter II). Disseminated tumor cells were well accessible for antibodies when this monoclonal antibody was injected intravenously (Chapter III).

Under various conditions successful tumor therapy was achieved with ascites fluid containing high titers of this monoclonal antibody (Chapter IV). The monoclonal antibody was found to be of the IgG2a subclass and is able to mediate both complement dependent cytolysis and antibody dependent cellular cytotoxicity. Our results favor the latter mechanism being potentiated by helper T cells (Chapter V and VI).

Since the host animals are considered to be fully immunocompetent, the usefulness of our model is largely determined by the properties of the tumor. In comparison with other models used for immunotherapy a number of advantages and disadvantages of this model will therefore be discussed.

We consider the following aspects of our model for immunotherapy as advantages:

- In vitro culture of the RMB-1 tumor offers the possibility to screen for antibody reactivity or cytotoxicity. Cultured tumor cells can be used at any time to inoculate animals.
- The permanent presence of the target antigen on the tumor cells is important to prevent selection of a subpopulation not expressing the appropriate antigen and to prevent escape from therapy as described by Young and Hakamori (1981) and by Badger

and Bernstein (1983). In our model the expression of antigens was due to the synthesis of virally encoded proteins. One of these proteins, detected by Western blotting techniques, was glycosylated and most probably expressed on the cell membrane. This gp50 may be related to gag glycoproteins expressed on Gross, Friend, Moloney and Rauscher virus infected cells (Ledbetter and Nowinski, 1977; Evans et al., 1977; Schultz et al., 1979; Edwards and Fan, 1980). By immunoperoxidase staining no p30 molecules could be detected (unpublished results), although these molecules are abundantly present in virus. This finding can be explained by assuming that either the proviral gene coding for p30 is deleted in RMB-1 cells or the transcripts coding for p30 may not be properly processed.

- The antigen density has been related to the effectiveness of immunotherapy. Densities correlate positively with successfull therapy (Herlyn et al., 1985). High densities ranged from 10^6 to 3-4 x 10^6 molecules per cell, whereas in our case the number of binding sites was limited to about 2 x 10^5 molecules per cell which is apparently sufficient for a therapeutic effect. We demonstrated, however, a twofold increase after incubation with interferon-γ (Chapter VI) which could mean that combination therapy may even improve the results so that still higher tumor doses can be eradicated.
- Binding of the target antigen by gp50 by IC5F5 monoclonal antibody does not affect tumor growth as shown by prolonged cocultivation with and without antibodies (Chapter IV). Masui et al. (1984), Vollmers et al. (1985) and Sauvage et al. (1987) described that inhibition of growth under influence of monoclonal antibodies can occur. Although the presence of such a mechanism may improve the response to therapy, the role of host effector mechanisms in these systems are more difficult to analyse.
- The RMB-1 tumor is immunogenic due to the presence of virally induced antigens. Although this may be regarded as a disadvantage if the lack of tumor-specific antigens in humans is taken into account, immunogenicity may also be an advantage if interaction between several mechanisms is studied. As shown in Chapter VI, this tumor is able to provoke a DTH reaction and the

results of our experiments in nude mice (Chapter V) and those of others (Kennel et al., 1985; Lamon et al., 1987) stress the importance of T cell involvement in virally induced tumors. This is apparently less obvious in xenografts on nude mice, which can be well treated by monoclonal antibodies (Herlyn and Koprowski, 1982; Adams et al., 1984). These findings suggest an important influence of the tumor cell type on the host. The possible interaction between T cells and an ADCC mechanism has been discussed already in Chapter V.

- Virus production by the tumor cells is, as already mentioned, not detectable (de Both et al., 1981), which is an advantage for immunotherapy. Circulating virus particles may block antibodies and may lead to the continuous infection of new target cells, especially when the antibodies do not neutralize the virus. Moreover the viral particles are able to induce immunosuppression along different ways (rev. Bendinelli et al., 1985).
- We have used disseminated tumor foci in our studies, since sero-immunotherapy may be especially useful for treatment of small quantities of residual tumor cells. This situation represents better the clinical situation, in which problems are often caused particularly by the presence of residual, disseminated tumor. Small foci can be managed more readily by immune effector mechanisms than large tumors and are in general well accessible for antibodies and effector cells (Chapter III and V). Large (subcutaneous) tumors, however, may lead to local effector cell deficiencies (Johnson et al., 1979), and may therefore better be treated by conventional therapy.

<u>The following items may be considered as disadvantages of this model:</u>

- It is clear that this model has a number of limitations. The potential value of our experimental results for human tumor treatment remains difficult to predict. Although adult human T cell leukemia is known to be caused by HTLV-1 and cervix or hepatocellular carcinoma is frequently associated with certain virus strains (Zur Hausen, 1980), most human cancers have no proven viral etiology. The obvious consequence is that a number

of interactions present in this model, may play only a minor role in the treatment of human malignancies.

- Tumors composed of leukemic cells may differ in biological behaviour to carcinomas which are the majority of human malignancies. The differences may be dependent on the preservation of certain functions of the cell type from which these tumors are derived and may for instance become manifest by the degree of stroma reaction, necrosis or cohaesiveness which they provoke. Theoretically such factors may have implications for therapy, since the local situation influences antibody and effector cell penetration. Another drawback of the use of leukemic cells as tumor model is sometimes the difficulty to discern the neoplastic cells from host effector cells, particularly in immunohistological analyses (Delwel et al., 1987).
- Spontaneous metastases lead to a particular distribution pattern of tumor cells in the host, which is dependent also on acquired properties of the tumor cells, and may be absent in their parental tumors. These factors may affect immunotherapy of metastases. The consequences of these factors can not be studied in our model since therapy concerns the elimination of multicentric outgrowth of RMB-1 tumor cells which appear not mutually different.
- In a number of tumors the vascularisation is increased by tumor-derived angiogenic factors. Subcutaneous RMB-1 tumors lack extensive vascularisation and show readily central necrosis. Poor vascularization may hamper penetration of effector cells and antibodies. Therefore in our model subcutaneous tumors would be less suitable for immunotherapy studies. In our immunotherapy experiments this factor is circumvented by the treatment of small disseminated tumor foci.
- If inoculated properly the RMB-1 tumor grows rapidly and kills the host within a short time. This rapid growth is of benefit in therapy studies by giving quick results. On the other hand, however, the critical, therapy-refractory period is soon reached and may counteract a fine tuning of the dose response regimens.

Our studies lead to the followong conclusions with regard to immunotherapy with monoclonal antibodies in the model employed in this thesis:

1) The Rauscher virus induced myeloid cell line is able to elicit an immune response in the syngeneic host. This allows the immortalization of syngeneic antibody producing cells by the hybridoma technology.
2) RMB-1 cells express virally encoded antigens on the cell surface, which could be used as tumor-specific antigens in an immunotherapy model.
3) Interferon-γ increases the number of monoclonal antibody binding sites, and may be therefore, apart from other aspects, useful for combination therapy.
4) Radioactive-labelled specific monoclonal antibodies bind preferentially to targets in vivo. Binding is particularly prominent in well-vascularized parts of the tumor.
5) Successful immunotherapy with specific monoclonal antibodies is possible in the immunocompetent host provided the tumor burden is smaller than $\pm$ 2-3 x 10^8 cells which is about 1% of body weight.
6) Initial high doses of antibody are required for successful therapy.
7) No detectable modulation of the target antigen occurs during therapy.
8) The presence of the complement factor C5 is not a prerequisite for successful immunotherapy.
9) ADCC plays an important role in the tumor destruction and is probably particularly mediated by macrophages.
10) Tumor destruction is T cell dependent possibly via a DTH response acting in an additive or a synergistic way with ADCC.

The latter item (10) needs some comment:
Phagocytosis of RMB-1 tumor cells may yield antigen presenting cells which are able to activate helper T cells. The helper T cells in turn may stimulate macrophages for direct killing of tumor cells. This (DTH) mechanism may coexist with ADCC, causing

both separately the elimination of tumor cells. A complex interaction of both mechanisms, however, is imaginable as well. According to this view the helper T cells may also activate macrophages for ADCC which further intensifies this mechanism. Some of these involved macrophages again may function as antigen presenting cells and may recruit new helper T cells. In this way the DTH reactivity will be amplified.

(For references: see Introduction)

Summary

The treatment of disseminated cancer is still a matter of radiotherapeutical and chemotherapeutical intervention. These modalities have their drawbacks by exerting detrimental effects on the host. The investigations on a more selective therapy based on the immune reactions against tumor cells may therefore be advantageous.

This thesis deals with the treatment of disseminated tumors with specific monoclonal antibodies.

In an experimental model we have used a Rauscher virus induced, non-producing murine myeloid cell line derived from BALB/c mice (RMB-1). This cell line carries tumor-restricted antigens, which are immunogenic in the syngeneic host. The generation and characterization of two specific monoclonal antibodies of respectively the IgG2a and IgG2b subclass are reported in Chapter II. These monoclonal antibodies are directed to virally encoded gag-related proteins, expressed on the transformed myeloid cells. This is established by Western blot analysis of virus and RMB-1 lysates whereas the tissue specificity is determined by immunoperoxidase studies on fetal and adult BALB/c tissues and the ultrastructural localization by immuno-electron microscopy. No cross-reactivity with normal tissues was seen.

The accessibility of radioactive-labelled monoclonal antibodies to disseminated tumors is proved in Chapter III by total body scanning and by autoradiography.

Since antibody clearance may be important to establish dose regimens in immunotherapeutical studies, these parameters were also measured in tumor-bearing and control mice and show a decrease of the initial serum radioactivity of about 50% and 90% in 24 hours for respectively IC5F5 and 4D2B4.

In Chapter IV the effects of syngeneic monoclonal antibody therapy on established disseminated tumors was investigated by the use of the IgG2a monoclonal antibody IC5F5. From survival curves of treated and control tumor-bearing mice substantial evidence is obtained for effective therapy if treatment is

started within 5 days after intravenous inoculation of tumor cells. Untreated mice died within 15-20 days. An initial high dose of monoclonal antibodies seems most relevant.

In Chapter V the underlying mechanisms were explored by means of immunotherapeutical studies in complement factor C5 deficient DBA/2 mice and T cell deficient BALB/c nude mice. These studies were accompanied by (immuno)histochemical analysis of the tumor infiltrates in treated and non-treated tumor-bearing BALB/c mice and by in vitro studies on complement dependent (CDC) and antibody mediated cellular cytotoxicity (ADCC). This study suggested the presence of ADCC, most likely mediated by macrophages, whereas a major role of complement dependent cytolysis appeared to be unlikely.

In Chapter VI additional studies are reported on a delayed-type hypersensitivity (DTH) elicited by the tumor using the foot swelling assay. The conclusions from these latter two studies (Chapter V and VI) indicated an important role for T cells in this model.

It is hypothesized that in our model a DTH reaction on the tumor causes a helper T cell mediated influx of macrophages around tumor foci which may lead to a more effective ADCC, resulting in a complete eradication of tumor cells. However, elimination of tumor cells by both mechanisms individually is not excluded. Future studies should delineate the relative contribution of both types of immune elimination of tumor cells, and whether these responses operate additively or synergistically.

Samenvatting

Voor de behandeling van gemetastaseerde tumoren wordt nog steeds gebruik gemaakt van radio- en chemotherapie. Deze behandelingsmethoden hebben echter nadelen door hun ernstige bijwerkingen. Onderzoek naar een meer selectieve behandelingsvorm gebaseerd op immuunreactiviteit gericht tegen tumoren kan hierin mogelijk verbetering brengen.

Dit proefschrift behandelt immunotherapie van muizen met metastasen m.b.v. specifieke monoclonale antilichamen. In een proefdier model is gebruik gemaakt van een door het Rauscher leukemie virus in BALB/c muizen geinduceerde myeloide cellijn (RMB-1) welke geen virus produceert. Deze cellijn draagt tumorspecifieke antigenen welke immunogeen zijn in de BALB/c muizen. De selectie en karakterisering van twee specifieke monoclonale antilichamen van respectievelijk de IgG2a en IgG2b subklasse wordt vermeld in Hoofdstuk II. Deze monoclonale antilichamen zijn gericht tegen viraal gecodeerde, gag-geassocieerde eiwitten, aanwezig op de getransformeerde myeloide cellen. Dit is vastgesteld door middel van Western blot analyse van virus en RMB-1 lysaten, terwijl de weefselspecificiteit is bepaald met behulp van immunoperoxidase studies op foetaal en volwassen BALB/c weefsels en de ultrastructurele localisatie door middel van immunoelectron microscopie.

De bereikbaarheid van de tumoren voor de radioactief gemerkte monoclonale antilichamen is aangetoond met behulp van 'total body scanning' en autoradiografie (Hoofdstuk III). Daar de klaring van antilichamen van belang kan zijn voor het bepalen van dosering schema's in immunotherapeutische studies, werden deze parameters eveneens gemeten in tumor dragende en controle muizen. De gemeten waarden tonen een daling van de initiële serum radioactiviteit van ongeveer 50% en 90% voor respectievelijk IC5F5 en 4D2B4 in 24 uur. In hoofdstuk IV werd de therapeutische werking onderzocht van syngene monoclonale antilichamen op gedissemineerde tumoren, gebruik makend van het monoclonale antilichaam IC5F5.

Uit overlevingscurves van behandelde en controle tumordragende muizen bleek een duidelijk therapeutisch effect indien de behandeling werd gestart binnen 5 dagen na intraveneuze tumor inoculatie. Een hoge aanvangsdosis lijkt vooral van belang. Onbehandelde muizen overleden na een tijdsduur van 15-20 dagen.

In hoofdstuk V werden de werkingsmechanismen onderzocht door middel van immunotherapeutische studies in C5 deficiente DBA/2 muizen en T cel deficiente BALB/c naakte muizen. Tevens werden (immuno)histochemische analyses van tumor infiltraten in tumordragende behandelde en onbehandelde BALB/c muizen verricht. Hiernaast werden in vitro studies met betrekking tot complement afhankelijke (CDC) en antilichaam afhankelijke cellulaire cytotoxiciteit (ADCC) uitgevoerd.

Deze studie maakte het belang van een antilichaam afhankelijke cellulaire cytotoxiciteit door middel van macrofagen aannemelijk, terwijl een belangrijke rol van complement afhankelijke cytolysis minder waarschijnlijk werd.

In hoofdstuk VI worden studies beschreven ten aanzien van de door de tumor geinduceerde vertraagd-type overgevoeligheidsreactie (DTH). Hiertoe werd gebruik gemaakt van een assay waarbij zwelling van de achterpoot wordt gemeten als gevolg van de immuun-respons na locale injectie van tumorcellen volgend op s.c. immunisatie.

De conclusie uit beide laatste studies suggereren een belangrijke rol van T cellen in dit model gedurende de therapie met monoclonale antilichamen.

Als hypothese wordt gedacht aan een door de tumor opgeroepen DTH reactie, waarbij via geactiveerde T helper cellen een influx van macrofagen rond de tumor ontstaat. Dit kan leiden tot een meer effectieve ADCC met totale eliminatie van tumor cellen. Vernietiging van tumor cellen via beide werkings mechanismen afzonderlijk kan echter niet worden uitgesloten. Toekomstige studies zullen het aandeel van beide mechanismen moeten afgrenzen en een synergistische of additieve werking van beiden moeten aantonen.

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Curriculum vitae

De auteur werd in 1950 geboren te Dordrecht. Na het behalen van het HBS-B diploma werd in 1968 begonnen met de studie geneeskunde te Utrecht, welke werd beeindigd in 1975. Aansluitend was de schrijver voor 1 jaar verbonden aan de afdelingen Chirurgie (Drs. P.G. Oe, Dr. H.M. Kluck en Drs. J.T. Tantua) en Gynaecologie/Verloskunde (Drs. P.J.A.M. Wijffels en Drs. W.L.A. Stroobands) van het St. Jozef Ziekenhuis te Kerkrade. Na afronding van de nationale tropencursus volgde vanaf 1977 een periode van 3 jaar waarin de auteur, via uitzending door Memisa, als arts verbonden was aan het Hôpital St. Jean de Dieu te Boko in de République Populaire du Bénin. Na terugkomst werd, na 9 maanden als artsassistent werkzaam te zijn geweest op de chirurgische afdeling van het Marien Hospital te Kevelaer in West-Duitsland (Dr. W. Schwanitz), in 1981 begonnen met de opleiding tot anaesthesist op de afdeling Anaesthesiologie van het Academisch Ziekenhuis te Leiden (hoofd Prof.Dr. J. Spierdijk). Deze opleiding werd echter na 1 jaar verwisseld voor de opleiding huisartsgeneeskunde aan het Universitair Huisartsen Instituut te Utrecht (toenmalig tijdelijk hoofd Dr. K. Gill). Na afronding hiervan was de schrijver werkzaam op de afdeling Klinische Pathologie van het Academisch Ziekenhuis Rotterdam-Dijkzigt (hoofd Prof.Dr. R.O. van der Heul) als artsassistent in opleiding. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht. Vanaf 1 juli 1988 is de schrijver verbonden als patholoog-anatoom aan het Pathologisch-Anatomisch Laboratorium voor Dordrecht en Omstreken.

Abbreviations

ADCC - antibody dependent cellular cytotoxicity
BCG - Bacillus Calmette - Guérin
BSA - bovine serum albumin
CDC - complement dependent cytotoxicity
CEA - carcino embryonic antigen
CTL - cytotoxic T lymphocyte
DNA - deoxyribonucleic acid
DTH - delayed-type hypersensitivity
ELISA - enzyme-linked immunosorbent assay
env - envelope (gene coding for envelope proteins)
FACS - fluorescence activated cell sorter
FCS - fetal calf serum
FITC - fluorescein isothiocyanate
FMR - Friend/Moloney/Rauscher
gag - group specific antigens (gene coding for core protein)
gp - glycoprotein
Gy - Gray
HBSS - Hanks' balanced salt solution
HIV - human immunodeficiency virus
HTLV - human T-cell leukemia virus
IF - immunofluorescence
IFN - interferon
Ig - immunoglobulin(s)
IL - interleukin
i.p. - intraperitoneally
i.v. - intravenously
Ka - association constant
L.I. - labeling index
LTR - long terminal repeat
Mab/Moab- monoclonal antibody
MCF - Mink cell focus-inducing
MHC - major histocompatibility complex
MLTC - mixed lymphocyte tumor culture
MST - median survival time
MSV - Moloney sarcoma virus

MuLV - Murine leukemia virus
NK - natural killer
n.t. - not tested
P - protein
PBS - phosphate buffered saline
pol - RNA-dependent DNA polymerase (gene coding for reverse transcriptase)
Pr - precursor protein
RED - Rauscher virus induced erythroid cell line of DBA/2 origin
RIA - radio-immuno assay(s)
RLD - Rauscher virus induced lymphoid cell line of DBA/2 origin
RMB - Rauscher virus induced myeloid cell line of Balb/c origin
RNA - ribonucleic acid
s.c. - subcutaneously
SEM - standard error of the mean
SFFV - spleen focus forming virus
Ts - suppressor T