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Natural Immunity in the Oncornavirus-infected Mouse[1]

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Summary

BALB/cfC3H females neonatally infected with mammary tumor virus (MTV) possess spleen cells capable of significant cytotoxic activity against target MTV-induced mammary tumor cells in microcytotoxicity assay. Spleen cells from supposedly MTV-free BALB/c females are also specifically reactive, as a result of horizontal transmission of MTV antigens. No quantitative differences in reactivity exist between females not susceptible to the development of mammary tumors (BALB/c) and those that may develop or already have tumors (multiparous BALB/cfC3H). However, there are qualitative differences in response between those at risk and those not at risk.

Introduction

A characteristic feature of natural infection with an oncornavirus is introduction of virus early in life; this has been clearly demonstrated in studies with experimental animals. This will complicate attempts to protect immunologically against the development of oncornavirus-induced tumors, since immune manipulations (nonspecific alteration or specific immunization) will produce modifications of existing immunity rather than establishment of response in an immunologically virgin individual.

The complexity of normal immune responses to oncornavirus antigens is well illustrated by our recent studies on BALB/cfC3H mice, productively infected with MTV[2] as a result of neonatal introduction of MTV via the mother's milk, and on genetically identical BALB/c mice, which do not receive MTV through the milk but which are exposed to MTV antigens in the mouse colony (3, 4, 6). Some of these studies have been reported in detail elsewhere (5–10, 14). Published results plus unpublished observations have been consolidated and summarized here.

Materials and Methods

Experimental Procedures. Methods used in these studies have been presented in detail (5–10, 14). Immune responses were assayed by the ability of spleen cells (with most of the macrophages removed) to show cytotoxic activity against target MTV-induced mammary tumor cells in a 2-day *in vitro* microcytotoxicity test and by the ability of serum to block or to enhance this cytotoxic activity. Specificity of detected responses was demonstrated by lack of reactivity of such spleen cell preparations against isogeneic MTV-free carcinogen-induced mammary tumor cells and isogeneic murine leukemia virus-producing mammary tumor cells (9).

Results

Immune Responses of BALB/cfC3H Females. Within the 1st month of life, spleen cell activity is detectable in neonatally infected BALB/cfC3H females (7). At 3 and 4 weeks of age some females may still be unresponsive, but by 8 weeks of age (and perhaps earlier) essentially all females are reactive (9). This reactivity is, however, of relatively low intensity; a statistically significant decrease in target cell survival has been found in only about two-thirds of the females tested (Table 1).

The response of BALB/cfC3H virgin females is also characterized by the presence of serum factors that completely block BALB/cfC3H T-cell spleen cell attack if target cells are pretreated with serum before addition of spleen cells (7, 9) or partially block cytotoxicity if spleen cells are pretreated (unpublished observations).

Cytotoxicity of spleen cells from BALB/cfC3H virgin females is dependent upon the presence of T-cells; it can be eliminated by pretreatment of spleen cells with antiserum against θ antigen, and it is not affected by pretreatment of spleen cells with aggregated γ-globulin (14).

Mammary tumor incidence in virgin BALB/cfC3H females is relatively low; in a recent test, only 7 of 23 females (30%) developed tumors. We have examined the spleen cell response of a few tumor-bearing virgin females; all showed a significant but still relatively low level of activity (7).

Although the intensity of the spleen cell response is apparently not much affected by the development of a tumor, another physiological event has a profound effect. After pregnancy and lactation, females possess spleen T-cells with significantly more reactivity (7). In addition, a new type of reactivity, antibody-dependent cell-mediated cytotoxicity, is now detectable (14). Two types of cells are needed to carry out the non-T-cell cytotoxic activity, an immunoglobulin-bearing non-T-cell lymphocyte that secretes antibody in culture (8), and an otherwise nonreactive cell that can be recruited to cytotoxic activity in the presence of specific antibody and antigen (5, 8). With an increasing number of pregnancies, spleen cell cytotoxic activity becomes even stronger (Table 1).

Mammary tumor development in multiparous

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[2] The abbreviations used are: MTV, mammary tumor virus; T-cell, thymus-derived lymphocyte.

Table 1

Activity against MTV-induced mammary tumor cells of spleen cell preparations from BALB/cfC3H females

Determination of average percentage of survival of target tumor cells in cultures incubated with spleen cells as compared to 100% survival in cell control cultures. The significance of these differences has been calculated from the actual cell numbers. This table was compiled from data in Refs. 5, 7, 9, 10, and 14.

	Significant decrease in target cell survival			Decrease in target cell survival not significant		
		% survival			% survival	
Spleen cell donors	No. of mice	Av.	Range	No. of mice	Av.	Range
3 wk old	5	88	86–92	3	98	93–101
4 wk old	2	90	88, 91	2	100	99, 100
5 wk old	2	90	87, 92			
8–32 wk old, virgin	23	86	74–94	12	92	84–97
Tumor-bearing, virgin, 12.5–14 mos. old	4	79	75–89			
Parous, 1 litter	5	56	48–73			
Parous, 2 litters	4	49	42–63			
Parous, 3 or more litters	10	41	33–46			
Tumor-bearing, multiparous	16	53	41–77			

BALB/cfC3H females is frequent, and tumors usually develop earlier than in virgin females. In breeding females of our BALB/cfC3H nucleus stock, we recently recorded a tumor incidence of 82%, at an average age of 7.9 months. We have detected no change in the pattern of spleen cell activity when mammary tumors first develop in multiparous females (7). However, during the growth of the tumor, the non-T-cell spleen cell activity becomes undetectable, and is not found in females bearing large progressively growing tumors (7, 14). Both cell types necessary for the antibody-dependent cell-mediated cytotoxic reaction are still present in the spleen, but they are considerably less frequent. The number of cells secreting specific antibody in culture is about one-tenth of that found in tumor-free multiparous females (8), and spleens of tumor-bearing females are a poor source of recruitable cells (Ref. 8; unpublished observations).

Blocking factor (effective against BALB/cfC3H T-cells) is still present in the serum of multiparous females bearing large tumors; it can easily be detected when the serum is used for pretreatment of isologous mammary tumor target cells (Table 2). In addition, 2 other serum components become detectable: (*a*) a blocking factor which can inhibit a non-T-cell cytotoxic activity; and (*b*) a tumor-specific antibody which recruits normal cells to cytotoxic activity (Ref. 5; unpublished observations).

The new blocking factor found in the sera of tumor-bearing females can protect pretreated target tumor cells from the specific spleen cell attack (non-T-cell) of BALB/c females. As yet, we do not know when this type of blocking activity arises; we have not tested for it in the sera of females bearing small tumors. However, we have examined the sera of multiparous but tumor-free females for this factor; we found it in only 1 of 11 tested (Table 2).

The tumor-specific recruiting antibody can be detected only if tumor-bearer serum is used to pretreat target tumor cells obtained from the same mouse. No blocking is detected if target cells are pretreated with autologous serum; instead, cytotoxic activity of spleen cells is increased and spleen cells that are not normally reactive become cytotoxic to the serum-pretreated target cells (5). Thus, the tumor-bearing female is reacting not only to MTV-associated antigens characteristic of all MTV-induced tumors of this type but also to an antigenicity specific for each individual tumor. The existence of such tumor-specific antigenicity, in addition to MTV-associated antigenicity, in some mammary tumors has been demonstrated *in vitro* (11) and in transplantation experiments (17). The tumor-specific recruiting antibodies (or complexes) are present early in the growth of a tumor and remain easily detectable as the tumor increases in size (unpublished observations).

Thus, the immune reactivity of the female neonatally infected with MTV changes considerably during her lifetime. Specific reactivity in serum and in spleen cells is detectable in all females after the 1st month or 2 of life. The response increases both quantitatively and qualitatively with parity, possibly as a response to viral replication in the mammary gland during lactation. With tumor development, 2 new serum components become detectable, but 1 spleen cell activity falls below detectable levels (Table 3).

Immune Responses of BALB/c Mice. Specific immune reactivity to MTV-associated antigens is not limited to those animals that receive MTV neonatally by milk transfer; spleen cells from adult BALB/c mice are also significantly reactive (Table 4).

BALB/c mice do not receive MTV by milk transfer. Genomically integrated MTV may be present (1, 2, 18), but we have no evidence for its expression in our subline. MTV has not been detected immunologically or by electron microscopic examination in the relatively rare mammary tumors that

Table 2

Summary of blocking activity detectable in the sera of BALB/cfC3H and BALB/c females

Target MTV-induced mammary tumor cells pretreated with serum before addition of spleen cells. This table was compiled from data in Refs. 5, 7, and 9 and from unpublished observations.

	Serum donor			
Spleen cell donor	BALB/cfC3H virgin adult	BALB/cfC3H multiparous	BALB/cfC3H multiparous tumor-bearing	BALB/c virgin adult
BALB/cfC3H virgin adult	++[a]	++	++	0
BALB/cfC3H multiparous tumor-bearing	++	++	++	0
BALB/c virgin adult	0	0[b]	++	+

[a] 0, no blocking; +, partial blocking, statistically significant; ++, complete blocking, statistically significant.

[b] Ten of 11 individual serum samples tested did not inhibit spleen cell reactivity; 1 of 11 blocked BALB/c spleen cell cytotoxicity completely (unpublished observations).

Table 3

Type of cytotoxic activity against MTV-induced mammary tumor target cells detectable in spleen cell preparations from BALB/cfC3H and BALB/c females

This table was compiled from data in Refs. 7 and 14.

Spleen cell donor	T-cell activity	Non-T-cell activity
BALB/cfC3H virgin	+[a]	0
BALB/cfC3H multiparous	+++	+
BALB/cfC3H multiparous with small tumor	+++	+
BALB/cfC3H multiparous with large tumor	+++	0
BALB/c 8 wk old	0	0
BALB/c 14 or more wk old	0	+++

[a] 0, no activity; +, detectable activity; +++, considerable and always significant activity.

appear in females of the BALB/c marked stock of the colony, although on very rare occasions MTV has been detected in unmarked supposedly BALB/c females.

However, although there is little or no evidence for the expression of MTV in mice of this subline, there is clear evidence of exposure to MTV antigens. Without exception, every adult BALB/c male or female that we have tested has possessed spleen cells capable of considerable and significant cytotoxic activity against MTV-induced BALB/cfC3H mammary tumor cells. This activity is specific; the spleen cells do not kill MTV-free carcinogen-induced mammary tumors or murine leukemia virus-producing mammary tumor cells (9). In addition, sera of these mice contain factors capable of blocking BALB/c spleen cell attack if target tumor cells are pretreated with serum (Table 2). The protection provided is only partial, but it is statistically significant (9).

Reactivity of BALB/c females to MTV-associated antigens is attained before the end of the 4th month of life; all females are reactive by 14 weeks of age (9) and the males are reactive even earlier. Spleen cells from a rare BALB/c female are positive at 10 or 13 weeks, none before that time (9).

The specific immune reactivity is a consequence of exposure to MTV or MTV antigens in the mouse colony; females raised in isolation remain unresponsive (6). Further, females raised in isolation become reactive after 4 weeks of exposure to a normal BALB/cfC3H female cagemate (6). BALB/c females raised in the mouse colony will also remain unresponsive to MTV antigens if their air supply is filtered, if their bedding and food are obtained from isolated supplies, and if they are handled only by someone who does not normally come into contact with MTV-infected mice; whether all or only some of these precautions are necessary has not yet been determined (unpublished observations). These isolation experiments provide neither positive nor negative evidence for the presence of genomically integrated MTV in BALB/c mice of our subline. They do, however, provide clear evidence that exposure to MTV antigens occurs naturally in the mouse colony and that an immune response is detectable as a result of this exposure.

Discussion

Although the picture of immune reactivity that we have developed is complex, it does not exhaust the possibilities. We have studied carefully mice of only 1 genotype; additional patterns of reactivity will undoubtedly become apparent when mice of other genotypes are studied. In most of our experiments, we have used only the spleen as a source of cytotoxic cells. Blood mononuclear cells and lymph node cells are also active (unpublished observations), but we have made no search for qualitative differences in cytotoxic cells from these sources. In addition, we have not yet examined the reactivity of all types of cells in the spleen, since we have routinely depleted the spleen cell preparations of most of the macrophages. Activity of macrophages against tumor cells of various types (nonspecifically if activated, specifically in the antibody-dependent cell-mediated cytotoxic reaction) has been described (12), and the activity of these cells against MTV-induced mammary tumors needs study. Further, we have used only 1 method of measuring immune reactivity, a 2-day assay of cytotoxicity using cultured tumor

Table 4

Activity against MTV-induced mammary tumor cells of spleen cell preparations from BALB/c females

Determination of average percentage of survival of target tumor cells in cultures incubated with spleen cells as compared to 100% survival in cell control cultures. The significance of these differences has been calculated from the actual cell numbers. This table was compiled from data in Refs. 6, 7, 9, 10, and 14.

	Significant decrease in target cell survival			Decrease in target cell survival not significant		
		% survival			% survival	
Spleen cell donors	No. of mice	Av.	Range	No. of mice	Av.	Range
8–9 wk old				7	104	100–109
10–13 wk old	2	57	49, 64	11	103	97–108
14 wk or older	41	56	40–80			

cells as target. Since assays differ in the type of immune reactivity that they reveal (13), other populations of immune cells may be detected when immunity is measured by techniques such as colony inhibition, release of isotope from target cells, inhibition of macrophage migration, or lymphoid cell blastogenesis (11, 13, 15, 16). Responses to additional tumor-associated antigens may also become detectable with other assays. Cytotoxic activity in our assay appears to be directed entirely against virion antigens, since it can be specifically blocked by pretreatment of the spleen cells with MTV virion preparations (10).

Although there may still be much to learn about "normal" immune responses to MTV-associated antigens, the information already available makes it clear that the response is complex. This complexity may be of some value in the development of diagnostic tests. With regard to the question posed to this conference, the feasibility of vaccines for tumor prevention, this complexity may provide complications in attempts to establish a uniform vaccination procedure that will be effective in providing protection to individuals with diverse "normal" patterns of immune reactivity to the antigens in question.

These results also have implications for attempts to assess human immune reactivity to oncornaviruses. We find that the age at exposure to MTV, or its antigens, and the route of introduction greatly affect both quantitative and qualitative aspects of the resulting immune response. However, if only the level of cytotoxicity is determined, no difference will be detected between a mouse that is not at risk (BALB/c) and one that may develop or already has a tumor (multiparous BALB/cfC3H). If the natural history of oncornavirus infection in the mouse has any parallel with the situation in the human, the choice of appropriate assays and controls for tests of specific immune responses of cancer patients and other humans may be difficult. We can anticipate many conflicting reports as human immune responses to oncornavirus-induced tumors are characterized, since the "normal controls" may well be as or more reactive (as the result of horizontal rather than vertical exposure to viral antigens) than the infected or tumor-bearing individual.

Immune responses to tumor cells, and also to allografts, are often characterized as T-cell responses; we detect a strong non-T-cell response as well (14). If non-T-cell responses are as effective *in vivo* as they are *in vitro*, then the question of altering an individual's immunity to increase its effectiveness will be a very complicated one. However, we must be cautious about assuming that the immune reactions detected *in vitro* are an accurate expression of the immune capabilities of the host (13). We do not know that the responses that we detect in spleen cell preparations are actually effective at the site of a developing mammary tumor or in preventing such a tumor from appearing. As already noted, the strongest *in vitro* responses that we find are characteristic of 2 distinctly different groups of females, those least likely to develop MTV-induced mammary tumors (virgin BALB/c) and those most likely to develop such tumors (multiparous BALB/cfC3H).

Although direct assessment of effective immunity may not be feasible, *in vitro* assays may still provide useful information in a characterized system. We find that, although mice at different levels of risk cannot be identified by the strength of the response, they can be identified by its quality. Thus, use of assays such as this may have some potential in prognosis.

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