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Immune Response in Preleukemic Mice

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Humoral and cellular immunity was assessed serially in preleukemic AKR mice and Gross virus-injected C3H/HeJ mice over a period of 10 to 12 months. Quantitative gamma globulin, hemagglutinin and hemolysin titers after immunization with sheep red blood cells, and macrophage-migration inhibition were determined. Normal production of gamma globulins as well as humoral antibodies was found in both of these experimental groups of mice, whereas macrophage-migration inhibition, which is believed to be a correlate of cellular immunity, appeared to be abnormal throughout the life of these animals, which were destined to develop a high incidence of lymphoma. Some aspects of this apparent dissociation between humoral and cellular immune response are discussed with regard to the mouse virus lymphoma system, primarily involving the thymus, which was used in these studies.

Impaired immune responsiveness in the presence of malignant disease poses the question of whether malignancy preferentially develops in individuals with impaired immunity. Virus-induced mouse leukemia is a useful model for answering this question, because one is dealing with a population in which the ultimate development of malignancy can be predicted. Several studies have been made in animals with the rapidly developing leukemias that follow the injection of Friend or Rauscher virus (3, 11, 18, 19, 22, 23). These diseases become overt only a few weeks after inoculation of the virus (2, 15). Immunological reactivity has been assessed by measuring humoral antibody titers, which have been shown to be depressed from shortly after virus inoculation to a stage of advanced disease. The short latent period between the inoculation of the virus and the appearance of overt malignant changes makes it difficult to evaluate true preleukemic immune responses in studies of these leukemias.

In lymphomas induced by the Moloney or Gross virus, the latent period from inoculation of the virus to the development of overt disease is several months. During much of this time, the morphology of the lymphoid tissues remains normal (9, 10). The infected animals ultimately show a high incidence of lymphoma, originating in the thymus and disseminating to lymphoid and other tissues (14). These diseases lend themselves to the study of immune response in a period when the animal is essentially normal, but destined to develop lymphoma. Cellular immunity has been shown to be depressed by measurement of skin-graft survival in Gross virus-infected preleukemic C3H mice (7) and in Gross virus-carrying preleukemic AKR mice (8). No serial studies have been done to follow the extent of impairment of cellular immunity at different times in the preleukemic period. Some controversy exists with regard to the impairment of humoral immunity in preleukemic Moloney and Gross virus-infected animals: some studies show depression of antibody formation before the appearance of overt lymphoma (4, 5, 21), whereas others deny an impairment of humoral immune response during the induction phase of this disease (16, 22).

In the present investigation, humoral and cellular immunity as well as immunoglobulins were studied in preleukemic, Gross virus-infected C3H/HeJ mice and in AKR mice, presumably congenitally infected with virus, at intervals during the prelymphoma period, to determine the extent and time of development of changes in the immune responsiveness of these animals.

MATERIALS AND METHODS

Animals. Two groups of mice from the inbred colonies maintained in this laboratory were used—the AKR strain, which naturally harbors Gross leukemogenic virus, transmits it to its progeny, and dies from lymphoma usually between the ages of 6 and 12 months; and the C3H/HeJ strain, which does not carry the virus but 50% of whose members die from

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lymphoma between the ages of 3 and 9 months when injected with Gross virus as newborns.

Mice were assigned to experiments in groups at the time of birth. Animals dying before the time of the experiment were autopsied to determine the cause of death. The thymus, lymph nodes, and spleen were examined. The diagnosis of lymphoma was made if a characteristic massive enlargement of the thymus was present. This was often accompanied by enlargement of the spleen, lymph nodes, and liver. This enlargement is caused by an infiltration of cells with the microscopic morphology of a lymphoblastic lymphoma. The words lymphoma and leukemia in this paper are used interchangeably to describe this disease.

Virus. The virus originally was supplied by L. Gross in the form of a leukemic $C3H_f/Bi$ mouse and has been maintained in this laboratory by repeated passage into 3- to 5-day-old AKR mice in which it produces a 100% incidence of accelerated lymphoma. Cell-free filtrates were made from leukemic tissues of AKR mice with accelerated lymphoma according to a procedure detailed previously (26). A 0.1-ml amount of filtrate was injected intraperitoneally into 3- to 5-day-old C3H/HeJ mice.

Protein studies. For quantitative protein determination, fresh tail blood was placed in nonheparinized capillary tubes and centrifuged; the serum was stored at −68 C until further processing. Total protein was measured by the method of Lowry et al. (13); 0.025-ml samples of serum were used. The amount of protein was read from a standard curve prepared from bovine serum albumin dilutions of known concentrations. Serum electrophoresis was done with a Phoroslide Micromethod (Millipore Corp., Bedford, Mass.). Electrophoresis strips were evaluated on a densitometer with electric integrator, and the different protein fractions were computed from the integrator's tracings.

Hemagglutinin and hemolysin titers. Five days prior to the collection of blood, mice were injected intraperitoneally with 0.3 ml of a 40% suspension of sheep red blood cells (SRBC). The animals were anesthetized and bled from the axillary artery. Prior to processing, serum was inactivated at 56 C for 30 min. Serial dilutions of individual sera were prepared by use of a microtiter apparatus. A 2% suspension of washed SRBC was used for hemagglutinin tests, and a 2% suspension of SRBC plus an equal volume of 5% guinea pig complement was used for hemolysin tests. Titers were read after 1 or 2 hr of incubation at 37 C for hemolysins and hemagglutinins, respectively, and recorded as the $\log_2$ of the last dilution of serum showing 1+ agglutination or hemolysis.

Macrophage-migration inhibition (MMI). Cellular type reactions were assessed by means of the MMI test as developed by George and Vaughan (12) and David et al. (6). Groups of mice were injected into the four footpads (0.05 ml in each foot) and the neck muscles (0.1 ml) with complete Freund's adjuvant 3 weeks prior to being studied for cellular immunity. At the time of the experiment, the animals were anesthetized and killed by bleeding from the axillary artery. By washing the peritoneal cavity several times with small amounts of cold saline through a small incision in the abdominal wall, peritoneal cell suspensions were obtained, containing 3×10^6 to 9×10^6 cells in 12 to 15 ml of saline, of which 60 to 90% were macrophages and 10 to 40% were lymphocytes. Lymph node cell suspensions were prepared from the same animals by grinding axillary and inguinal lymph nodes in a tissue grinder with cold saline, and 0.2 to 0.5 ml of this lymph node cell suspension containing 2×10^6 to 5×10^6 cells was added to the peritoneal cell suspension. The cell mixture was then centrifuged at 900 rev/min for 6 min; the supernatant was removed, and the cells were washed twice with cold Eagle's minimal essential medium. After the second washing, the cells were suspended in a small amount of the medium and placed into two to four capillary tubes 0.7 mm in inner diameter and 75 mm long. These tubes were sealed with plastic clay, and the cells were sedimented by centrifugation. The tubes were then cut at the cell-fluid interphase, and the cell-containing ends were placed into two Sykes-Moore culture chambers, one of which was filled with 15 μg of PPD (tuberculin, purified protein derivative) in Eagle's medium; the other was filled with Eagle's medium only, to serve as a control. The prepared chambers were incubated for 18 to 24 hr at 37 C. At that time, macrophage migration was observed under a dissecting microscope, the samples were photographed, and two diameters of the migration area, which had the shape of an elipse, were measured on black and white enlargements (× 35). The results are expressed as the percentage of migration area (obtained by multiplying the two diameters) in the antigen-containing chamber, based on the full migration area in the uninhibited control chamber as 100%.

RESULTS

Mortality from leukemia. Data from 168 AKR mice showed that the majority of the animals die with lymphoma between the ages of 7 and 11 months, and that by the age of 18 months 100% of these animals are dead from lymphoma. Mortality from lymphoma of C3H/HeJ mice was calculated for the 206 experimental animals used in this study. Between the ages of 3 and 10 months, about 50% of virus-injected C3H mice died from leukemia. These data compare very well with previous results obtained in this laboratory (26). Lymphoma did not develop in the breeding colony of C3H/HeJ mice during the first year of life.

Protein studies. Albumin and protein fractions of serum were determined quantitatively from serum electrophoresis and total protein measurements. As shown in Table 1, there were no significant differences between the serum levels of albumin and globulin in the three groups of animals studied. Similarly, total protein and gamma globulins in normal and virus-injected C3H/HeJ

TABLE 1. *Summary of serial serum protein studies in normal C3H/HeJ, virus-injected C3H/HeJ, and AKR mice*

Animal group	No. of animals	Total protein	Gamma globulins	Albumin	Globulins			
					α_1	α_{2-3}	β	γ
		mg/100 ml	*mg/100 ml*	%	%	%	%	%
C3H/HeJ normal								
1.5 months	14	4.8	0.13	40.4	18.0	17.1	20.5	2.7
2.5 months	11	6.2	0.35	34.1	15.5	13.2	23.9	5.7
4 months	8	6.0	0.34	40.6	13.5	17.0	22.9	5.7
7 months	6	6.0	0.47	34.9	17.4	14.4	25.3	7.9
10 months	10	6.6	0.42	40.2	13.5	13.2	27.6	6.3
12 months	10	5.1	0.36	37.8	15.1	14.4	26.0	6.6
C3H/HeJ virus-injected								
1.5 months	12	5.8	0.10	47.6	13.5	14.8	22.0	1.7
2.5 months	12	6.0	0.45	36.3	18.6	13.0	24.8	7.5
4 months	11	6.5	0.45	37.3	15.0	15.9	24.6	6.9
7 months	9	7.2	0.53	35.2	18.0	16.2	23.2	7.4
10 months	7	6.4	0.42	36.2	17.6	13.6	26.2	6.5
12 months	6	7.4	0.38	39.6	15.0	14.9	27.1	4.4
AKR								
1.5 months	7	4.7	0.11	41.5	17.9	16.5	22.0	2.4
2.5 months	11	5.1	0.29	41.4	17.0	16.6	19.1	5.7
4 months	13	5.9	0.34	37.1	13.9	16.3	25.5	5.8
7 months	11	7.0	0.39	41.7	17.3	13.6	21.7	5.6
10 months	7	5.4	0.33	36.8	21.1	12.2	23.5	6.2
12 months	2	5.2	0.14	40.0	24.6	11.1	21.6	2.7

mice showed no significant or consistent differences. However, in AKR mice the protein and gamma globulin levels appeared to be somewhat lower than in C3H/HeJ mice. This most likely was due to the difference in strain and may not be related to the presence of virus. Low Gamma globulin levels were found in 12-month-old AKR mice with lymphoma.

Hemagglutinin and hemolysin titers. In Fig. 1, the mean values for hemagglutinin and hemolysin titers of AKR mice and virus-injected and normal C3H mice are presented. No specific alteration in the immune response to SRBC could be demonstrated in animals with natural or induced viral infection. At 12 months, reduced titers were found in two AKR mice with generalized lymphoma, and one mouse with lymphoma had a normal immune response.

MMI. The results of the study of cellular immune response as assessed with the MMI test are represented in Fig. 2 and 3. The inhibition of macrophage migration is due to the production of MMI factor by immunologically stimulated lymphocytes (1). This is a qualitative rather than a quantitative method, which is dependent on the reactivity of a given animal to a specific antigen. The normal range of the results appeared to be quite wide, and individual animals were not

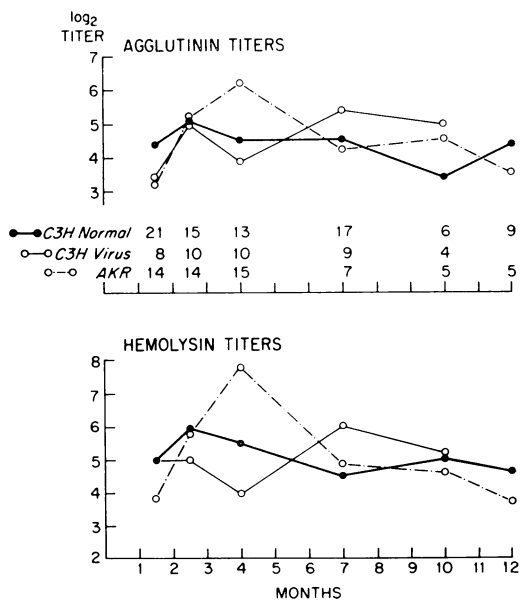

FIG. 1. *Means of sheep red blood cell hemagglutinin and hemolysin titers in untreated C3H/HeJ mice, Gross virus-injected C3H/HeJ mice, and preleukemic AKR mice at different ages. The numerals refer to the number of animals studied in each group.*

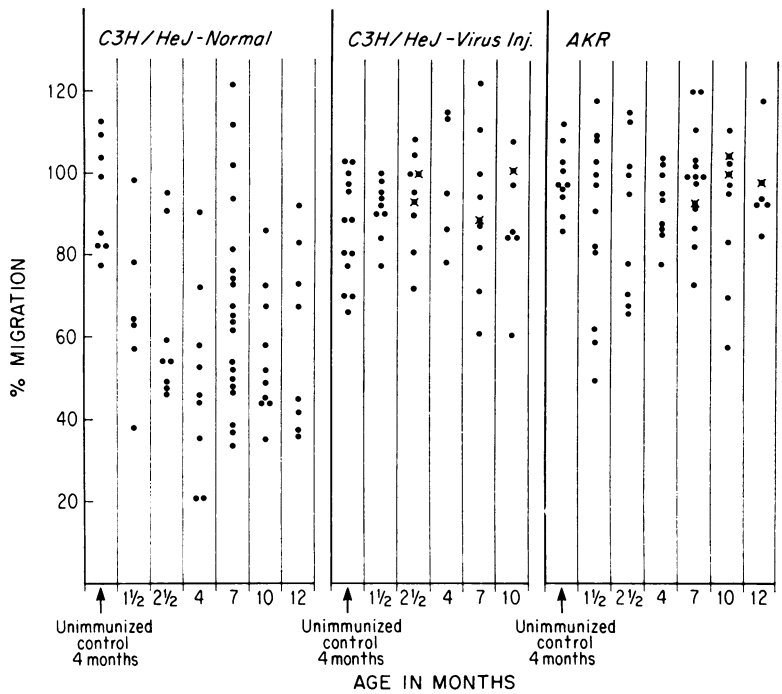

FIG. 2. *Macrophage migration in Freund's adjuvant-immunized and unimmunized normal C3H/HeJ mice, virus-injected C3H/HeJ mice, and preleukemic AKR mice. Each point represents the value for an individual animal. Crossed points indicate animals with macroscopic evidence of lymphoma at the time of the macrophage migration study.*

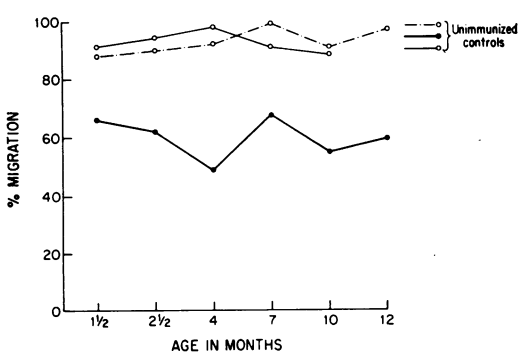

FIG. 3. *Means of the data presented in the scattergram in Fig. 2, comparing Freund's adjuvant-immunized mice in the three groups with 4-month-old unimmunized controls. Symbols:* O-·-·O, *AKR mice;* O—O, *virus-injected C3H/HeJ mice;* ●—●, *normal C3H/HeJ mice.*

necessarily representative of a whole group. The scattergram in Fig. 2 demonstrates the wide range of values found. However, when mean values were compared (Fig. 3), it was clear that macrophage migration was inhibited in immunized, normal C3H/HeJ mice in the presence of antigen throughout life to an average level of 60%. Such inhibition could not be demonstrated in preleukemic AKR mice or in C3H/HeJ mice given neonatal inoculation of the Gross leukemogenic virus. In these animals, macrophage migration was similar to that found in the unimmunized controls of the same experimental group. This difference between normal and preleukemic mice persisted throughout the observation period from the age of 1.5 to 10 or 12 months. This difference in MMI between normal animals and those with naturally occurring or induced virus infection for most points was highly significant as calculated with the Student *t* test (Table 2).

DISCUSSION

This study was concerned with the immunological response during the latent phase of tumor development in potentially leukemic mice. Immunity was tested in two basically different strains of animals. Mice of the AKR strain transmit causative virus from mother to offspring. Eventually, lymphoma develops in 100% of the animals. Mice of the C3H/HeJ strain develop

TABLE 2. *Significance of difference in macrophage migration between animals with natural and induced Gross virus infection and normal animals*

Experimental groups compared	P^a					
	1.5 months	2.5 months	4 months	7 months	10 months	12 months
C3H normals/C3H virus-injected	<0.005	<0.001	<0.005	<0.02	<0.001	
C3H normals/AKR	<0.01	<0.02	<0.001	<0.001	<0.001	<0.005

[a] Determined by the Student *t* test.

lymphoma only when inoculated with Gross virus and then with a lower incidence, but shorter latent period, than that found in the "spontaneous" disease of the AKR strain. Lymphoma, in these two situations, is known to develop first in the thymus, from which the malignant cells disseminate to produce a generalized disease. Metcalf demonstrated that transplantation of AKR preleukemic thymus into young AKR mice did not induce leukemia unless lymphoma cells could be shown to be present in the graft at the time of transplantation, and such cells could not be found earlier than 4 or 6 weeks before death from lymphoma (14). Thus, the assumptions can be made that AKR mice are free from lymphoma cells before the age of 4 months, and that malignant cells are probably not present in virus-injected C3H mice until after 2 months of age. Therefore, observations made in the early months of life in AKR and virus-inoculated C3H/HeJ mice are considered to be in animals with established viral infection but without lymphoma cells.

In the present study, no impairment of humoral immunity, as assessed indirectly by quantitative gamma globulin determination and directly by hemagglutinin and hemolysin responses to SRBC, was found at any age. As mentioned previously, impaired humoral antibody responses have been found during the preleukemic period after inoculation of Friend and Rauscher viruses. Because the viruses produce almost immediate morphological alterations in the lymphoid system and cause leukemia within a few weeks after inoculation, their effects on humoral immunity cannot be directly compared with the long latent period system used in the present study. In Moloney virus-infected rats, Cremer showed an increasing depression of gamma globulin levels and a moderate though consistent reduction in hemolysin and hemagglutinin titers during the preleukemic period (4, 5). Peterson and co-workers, using a Gross virus-infected C3H strain, also showed some impairment of antibody production against T2 phage (21). On the other hand, Salaman and Wedderburn did not find defective production of hemagglutinins in Moloney virus-infected preleukemic Balb/c mice (22). Metcalf and Moulds, in a similar study of humoral immunity in preleukemic AKR mice, reported normal formation of the hemolytic plaques by spleen cells and normal hemagglutinin or hemolysin titers after stimulation of these animals with SRBC, unless obvious lymphoma was present (16).

In the present report, the MMI test when adapted to mice demonstrated that the MMI factor is depressed in the prelymphoma period in animals with both virus-induced and spontaneous disease. If, as has been shown by others (6, 12, 24, 25), MMI is a true correlate of delayed hypersensitivity or, in other terms, cellular immunity, these results agree with published studies (7, 8) showing impaired cellular immune response in preleukemic C3H and AKR mice as assessed by prolonged skin allograft survival.

The finding of an impaired cellular immune response but intact humoral immunity in the mice used in the present studies should not be unexpected. The thymus has been shown to be necessary for the development of normal cellular immunity (17), and this same organ is the target for the development of lymphoma in the two host systems studied. This fact allows for speculation as to the mechanisms of lymphomagenesis. It could be postulated that impaired cellular immunity is the result of a viral attack on thymus cells. This might then cause a derangement of thymus function which, occurring over a period of time, could ultimately result in lymphoma. Alternatively, the fact that impaired cellular immunity occurs long before overt lymphoma suggests a dual role for the virus: immunosuppression and malignant transformation. Tumor-specific antigens have been demonstrated in Gross virus-induced lymphomas (20); therefore, lymphoma could result when the virus-transformed malignant cell found itself in an environment of impaired host cellular immunity. The present studies do not provide sufficient evidence to allow conclusions along these lines, but they do show

that specific functional changes in the immune mechanism occur in the prelymphoma period that must be related to the organ in which the malignant disease is ultimately found.

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