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Cell-Mediated Immune Responses of Preleukemic AKR Mice

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Splenic lymphocytes from preleukemic AKR mice are capable of participating in various cell-mediated immune responses. Spleen cells from AKR mice aged 1 to 10 months produced a significant graft-versus-host reaction when injected into 8-day-old AKR × C57Bl/6 F_1 hybrids. Splenic lymphocytes from similarly aged mice were also capable of responding to allogenic stimulation in a mixed lymphocyte reaction. AKR mice aged 1 to 10 months developed a contact sensitivity response to picryl chloride. It was concluded that mice of this high-leukemic strain have a competent cell-mediated immune system and there is no depression of immunity during the preleukemic period.

Depression of cell-mediated immunity may be associated with the expression of cancer. The increased occurrence of malignancies in both man and laboratory animals after immunosuppressive therapy lends support to this hypothesis (16). Furthermore, infection of susceptible mice with Friend leukemia virus results in the depression of cell-mediated immunity prior to the onset of malignancy (6, 13). However, in strains of mice which develop spontaneous lymphoid leukemia, such as the AKR strain, there are conflicting reports on the cell-mediated immune capacity during the preleukemic period. Female AKR mice were shown to have a decreased ability to reject syngeneic male skin as compared to C57Bl/6 mice (3). Migration inhibition factor production was shown to be impaired throughout the life span of AKR mice (5), and AKR spleen cells exhibited a reduced ability to induce a graft-versus-host reaction (GVHR) (9). On the other hand, AKR mice were shown to be capable of rejecting allografted lymphoma cells (8), sarcoma 180 (4), and allografted skin from mice of the same sex (10). Hargis and Malkiel (7) reported spleen cells from 1- to 11-month-old AKR mice were capable of inducing a GVHR in newborn CFW mice. In the present investigation, the cellular immunocapacity of preleukemic AKR mice was studied to ascertain if a depression in cell-mediated immunity was a necessary prerequisite for the onset of the disease. The in vivo models of cellular immunity, GVHR and contact sensitivity, were utilized as was the in vitro correlate of GVHR, the mixed leukocyte reaction (MLR).

MATERIALS AND METHODS

Animals. AKR and C57Bl/6 were originally obtained from the Jackson Laboratory, Bar Harbor, Me., and subsequently maintained in this laboratory by strict brother-sister mating. The incidence of generalized lymphoma was 87% in AKR mice with the average age of death 9.4 months. All mice were fed Wayne sterilizable Lab-Blox and water ad libitum.

GVH reaction. Donor mice were sacrificed and spleens were removed aseptically. Single-cell suspensions were prepared by passing the spleens through a 100-mesh stainless-steel wire screen into cold medium 199 (M199) with Earle modified salts (GIBCO, Grand Island, N.Y.). The cells were washed three times with cold M199 and collected each time by low-speed centrifugation. A total leukocyte count was performed by employing a hemocytometer, and viability was determined by trypan blue exclusion. Eight-day-old AKR × C57Bl/6 F_1 (AKB6F_1) mice were injected intraperitoneally with various concentrations of viable spleen cells in 0.1 ml of M199 (Fig. 1). Since an inoculum of 10×10^6 viable spleen cells elicited splenic indexes greater than two, this cell concentration was used throughout. In all experiments at least two uninjected litter mates served as controls. Eight days postinjection, the spleen weight assay for GVH reactivity was performed by the method of Simonsen and Jensen (15). A splenic index of greater than 1.3 is considered positive.

MLR. Donor mice were sacrificed and the spleens were removed aseptically. Single-cell suspensions were prepared as before. Stimulator cells were obtained by treatment with mitomycin C (MC) (Nutritional Biochemicals) at the concentration of 20 μg of MC per 10^6 cells for 30 min at 37 C with agitation. After incubation, stimulator cells were washed three times with M199 and collected each time by low-speed centrifugation (15 min at 275 × *g*). Stimulator cells (10^6) and responder cells (10^6) were cultured in glass tubes (13 by 100 mm) with metal closures in 1 ml of M199 supplemented with 20% fetal calf serum and 100 units of penicillin per ml and 100 μg of streptomycin per ml. All cultures were incubated in a 5% CO_2 atmosphere at 37 C for 5 days. During the last 24 h of incubation of 1 μCi of ^{3}H-labeled thymidine (New

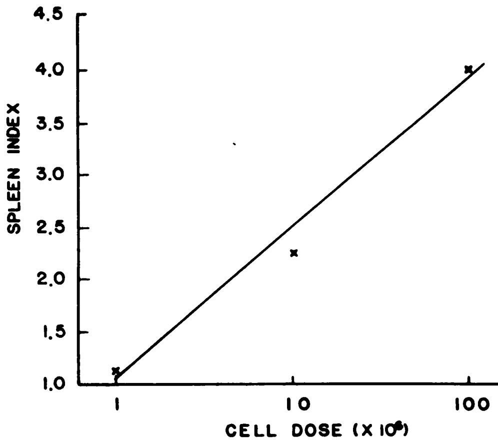

FIG. 1. *Dose response curve from injection of adult AKR spleen cells into 8-day AKB6 (AKR × C57Bl/6) F_1 mice.*

England Nuclear Corp., specific activity 20 Ci/mmol) was added per culture. After the incubation period, 2 ml of phosphate-buffered saline (PBS) with 2 mg of nonradioactive thymidine per ml was added to each culture. The cells of each tube were collected on a membrane filter (0.45-μm pore size; Millipore Corp.) and washed twice with 10 ml of PBS, twice with 5 ml of cold 5% trichloroacetic acid, and once with 5 ml of absolute ethanol. The filter pads were dried under an infrared heat lamp for 30 min and placed in scintillation vials. A 10-ml amount of scintillation cocktail (scintillation-grade toluene with 5 g of 2,5-diphenyloxazole per liter and 0.3 g of 1,4-bis-(5-phenyloxazolyl)-benzene per liter (from New England Nuclear Corp.) was added to each vial. All vials were counted for 10 min in a Packard Tri-Carb liquid scintillation counter model 3375. Results are expressed as the counts per minute and standard deviation of quadruplicate samples. The stimulation index was calculated by subtracting the stimulator cell counts per minute from the stimulator-responder cell mixture counts per minute and dividing by the responder cell counts per minute.

Contact sensitivity. A modification of the technique of Asherson and Ptak (1) was used for determining contact sensitivity in AKR mice aged 1, 3, 5, 7, and 10 months. The mice were anesthesized with sodium pentobarbital, the abdomen was shaved, and they were immunized by painting 0.1 ml of an 8% picryl chloride in acetone solution on the shaved area. Six days post-sensitization, all mice were challenged with 1% picryl chloride in acetone on the right ear, whereas the left ear received acetone alone. Both sides of each ear were painted with 3 drops of the appropriate solution (0.006 ml) from a 27-gauge needle. The thicknesses of both ears were measured with an engineer's micrometer at 0, 4, 12, 24, 48, and 72 h after challenge. Thicknesses were expressed in units of 10^{-3} mm, with the normal ear ranging from 23 to 30 units. Significance was determined with Student's *t* test.

RESULTS

GVH reaction. The strain combination AKR × C57Bl/6 ($AKB6F_1$) was chosen to maximize the histocompatibility difference, AKR being $H\text{-}2^k$ and C57Bl/6 being $H\text{-}2^b$, and establish a reproducible graft-versus-host system as described by Simonsen and Jensen (15). This combination also minimizes the possibility of transferred Gross virus inducing a lymphoma since it has been demonstrated that $AKB6F_1$ mice have a low incidence of spontaneous leukemia (11). At least 15 experimental $AKB6F_1$ neonates representing four different litters were recipients of either AKR or C57Bl/6 spleen cells. These donor cells were from animals aged 1, 3, 5, 7, and 10 months of age. All AKR donor cells were from animals which showed no gross signs of leukemia, absence of thymus and lymph node enlargement and splenomegaly, at the time of spleen cell isolation. Figure 2 illustrates the results of GVHR induced by either AKR or C57Bl/6 spleen cells. AKR splenic lymphocytes at all ages tested were capable of inducing a significant GVHR (>1.3) in F_1 recipients. The ability of AKR spleen cells to mount a GVHR increased from 1 to 5 months, peaked at 5 to 7 months, and decreased to the initial level by 10 months of age. When C57Bl/6 spleen cells were employed to induce a GVHR, the response curve was similar to that seen with AKR cells. However, the magnitude of the C57Bl/6 response was higher than the AKR response.

MLR. One-way mixed leukocyte reactions were used as an in vitro correlate of the GVHR. Table 1 illustrates the response when AKR spleen cells from mice of various ages and showing no gross signs of lymphoma are reacted with 2-month-old C57Bl/6 mitomycin C-treated spleen cells. The values depicted are the mean, standard deviation, and stimulation index of four replicate samples in which the responder cells were pooled from at least four mice of the

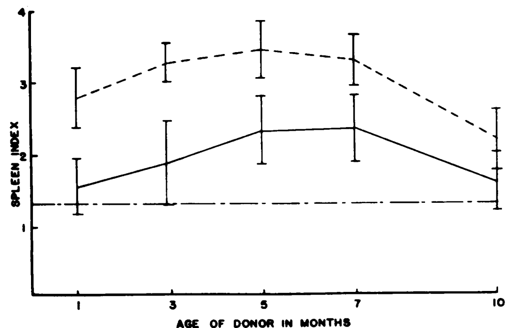

FIG. 2. *Comparison of graft-versus-host response in AKB6 (AKR × C57Bl/6) F_1 mice from injection of various aged AKR (——) or C57Bl/6 (- - -) spleen cells. The vertical bars represent one standard deviation of the mean. Horizontal line (-·-), theoretical splenic index of 1.3 which is indicative of a GVHR.*

TABLE 1. *Results of a one-way MLI employing AKR spleen cells as responder cells*

Age (months)	Unstimulated[a] (counts/min)	Stimulated[a] (counts/min)	Stimulation index[b]
1	3,690.4 ± 470[c]	6,211.2 ± 255	1.68
	2,082.6 ± 311	3,062.0 ± 455.1	1.47
	491.2 ± 16	810.4 ± 198.9	1.64
3	585.9 ± 146	1,807.8 ± 893	3.08
	460.4 ± 52	2,959.1 ± 120	6.42
	1,724.1 ± 120	6,164.5 ± 309	3.57
5	472.5 ± 40	1,690.9 ± 473	3.57
	216.6 ± 80.3	605.0 ± 317.4	2.79
	497.5 ± 50.2	2,665.9 ± 300.5	4.90
7	1,582.2 ± 238	8,219.2 ± 542	5.19
	461.1 ± 130	2,627.9 ± 263	5.69
	1,387.7 ± 158.9	5,585.3 ± 620.4	3.86
10	300.9 ± 38	1,625.9 ± 259	5.40

[a] Arithmetic mean and standard deviation of 4 replicate samples.

[b] $\frac{\text{(Stimulator counts per minute + responder counts per minute) − stimulator counts per minute}}{\text{responder counts per minute}}$

The mean of four replicate samples were utilized in all calculations.

[c] Each set of data (unstimulated counts per minute, stimulated counts per minute, and stimulation index) represents one experiment.

stated age. At 1-month of age the stimulation index of the AKR spleen cells indicated only a slight response. However, by 3 months of age the stimulation indexes had doubled and the heightened response remained at those levels throughout the ages tested. The responses of similarly aged C57Bl/6 spleen cells to mitomycin C-treated AKR cells are illustrated in Table 2. There was no significant difference between the stimulation indexes of spleen cells from 1-, 3-, 5-, 7-, and 10-month-old C57Bl/6 mice.

Contact sensitivity. Preliminary studies similar to those of Asherson and Ptak (1) were carried out to determine if a single topical immunization with picryl chloride was sufficient to induce contact sensitivity in AKR mice. It was found that 0.1 ml of 8% picryl chloride in acetone applied topically induced a marked reaction when the animals were challenged 6 days after immunization. Ten immunized and five nonimmunized AKR mice at each age group were utilized. Preliminary experiments showed a contact sensitivity response as 12 h, reaching a maximum at 24 h, and decreasing to near normal levels by 72 h. When AKR mice aged 1, 3, 5, 7, and 10 months were assayed for contact sensitivity, the 24-h response was fully manifest in 1-month-old mice (Table 3). This response remained at about the same level at all ages tested.

TABLE 2. *Results of a one-way MLI employing C57Bl/6 spleen cells as responder cells*

Age (months)	Unstimulated[a] (counts/min)	Stimulated[a] (counts/min)	Stimulation index[b]
1	1,049.1 ± 227[c]	4,226.6 ± 210	4.02
	340.9 ± 158.5	1,360.6 ± 286.7	3.99
3	593.6 ± 226	3,346.5 ± 253	5.63
	690.8 ± 286	3,675.1 ± 299	5.32
5	381.2 ± 33.4	1,911.5 ± 420.7	5.01
7	778.8 ± 36	4,131.7 ± 632	5.30
	665.6 ± 178	3,712.8 ± 263	5.24
10	184.6 ± 6.9	959.0 ± 224	5.19
	458.8 ± 180	1,408.1 ± 554	3.06

[a] Arithmetic mean and standard deviation of 4 replicate samples.

[b] $\frac{\text{(Stimulator counts per minute + responder counts per minute) − stimulator counts per minute}}{\text{responder counts per minute}}$

The mean of four replicate samples were utilized in all calculations.

[c] Each set of data (unstimulated counts per minute, stimulated counts per minute, and stimulation index) represents one experiment.

TABLE 3. *Increase in AKR ear thickness at 24 h after challenge with picryl chloride*

Age (months)	Group	Mean[a]	Range[b]	Significance[c]
1	Control	2.92	1.27–11.43	$P < 0.001$
	Experimental	15.83	8.79–20.32	
3	Control	3.51	0.00–8.79	$P < 0.001$
	Experimental	12.27	6.25–20.32	
5	Control	4.13	0.00–7.52	$P < 0.001$
	Experimental	12.91	7.52–19.05	
7	Control	4.62	0.00–11.43	$P < 0.001$
	Experimental	15.87	8.79–19.05	
10	Control	5.56	2.54–12.70	$P < 0.001$
	Experimental	16.36	13.97–21.59	

[a] Arithmetic mean of at least 10 mice.
[b] Minimum and maximum increase in ear thickness.
[c] Significance determined by Student's two-tailed t test.

DISCUSSION

Control of aberrant cells and resulting malignancies has been attributed to the immunologic surveillance system (2). Deficiencies in the surveillance mechanism whether natural or artificial (16) lead to an increased incidence of de

novo tumors. Experimentation utilizing passaged leukemia viruses has also supported this concept. Susceptible mice infected with Friend leukemia virus exhibited a marked depression of delayed-type hypersensitivity to purified protein derivative (PPD) and depression of migration inhibition factor prior to the onset of leukemia (13). A similar immune depression was observed following infection with Rauscher leukemia virus in susceptible mice (12).

To date, however, there is very little data concerning cellular immune competence prior to the onset of spontaneously arising murine leukemias. Mice of the AKR strain naturally harbor vertically transmitted Gross virus and spontaneously develop a lymphoid leukemia of thymic origin. The initial thymoma becomes evident during the preleukemic period (4 to 6 months of age) and usually becomes disseminated (leukemic period), with 85 to 90% of the mice dying by 11 months of age. If a breakdown of the immune surveillance system were a prerequisite for viral-induced cancer in the AKR mouse, it should occur during the preleukemic period. Our experiments, however, have shown that there is no observable decrease in cell-mediated immunity in AKR mice during the preleukemic period.

Spleen cells from AKR donor mice aged 1 to 10 months were able to induce a significant GVHR in AKR × C57Bl/6 F_1 hybrids. Similar results were obtained by Hargis and Malkiel (7). They showed AKR spleen cells from donors aged 1 through 11 months were capable of inducing a significant GVHR in newborn CFW mice. In contrast, AKR spleen cells produced only marginal GVHR when injected into SJL × AKR F_1 hybrids (9). Also, there was no correlation between the degree of GVHR and varying lymphocyte concentration within this strain combination. This apparent lack of reactivity may be due to the strain combination since AKR cells were able to induce GVHR in AKR × C57Bl/6 F_1 hybrids (Fig. 1) and in AKR × DBA F_1 hybrids (14). When donor AKR cell concentrations were increased, there was an concomitant increase in splenic index (Fig. 1) which was not present when AKR spleen cells were injected into SJL × AKR F_1 hybrids (9).

When the in vitro correlate of GVHR, the MLR, was employed to ascertain cellular immune reactivity, there was no diminution of this response during the preleukemic period. AKR spleen cells from donors aged 3 through 10 months responded to allogenic stimulation as shown by [^{3}H]thymidine incorporation. Thus, in both an in vivo and in vitro system preleukemic AKR spleen cells were capable of responding to alloantigens.

The contact sensitivity response to picryl chloride is also a measure of cell-mediated immunity (12). Mice of the AKR strain aged 1 through 10 months exhibited a significant contact sensitivity response to picryl chloride. The ability of spleen cells from AKR mice aged 7 and 10 months to induce a significant GVHR, MLR, and contact sensitivity response may be due to the selection of leukemia-resistant mice. The mice from the younger age groups would, on the other hand, have a larger percentage of leukemia-prone animals. Thus, the present data do not support the concept that a depression of cell-mediated immunity (immunological surveillance) is a prerequisite for the preleukemic state in AKR mice. Further experimentation is being conducted to elucidate the interaction of the Gross virus, thymus function, and cell-mediated immunity on the induction of lymphoid leukemia in AKR mice.

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