

Lysis of leukemia cells by spleen cells of normal mice

(natural immunity/AKR leukemia/immune surveillance/cell-mediated lysis/Cr release assay)

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Communicated by Frank J. Dixon, April 10, 1975

ABSTRACT Spleen cells from 2- to 3-month-old normal mice of some strains having a low incidence of spontaneous leukemia were found to lyse cells of the spontaneous AKR leukemia K36 in the ^{51}Cr release assay. Incubation of ^{51}Cr -labeled AKR K36 cells with spleen cells from normal C57BL/6, C57L, C57BL/10, and RF mice resulted in the release of significantly more ^{51}Cr than that released in the presence of medium alone. In contrast, ^{51}Cr released from AKR K36 cells after incubation with spleen cells from mice of the high leukemic strains AKR and C58 was less than that released spontaneously.

The results of competitive inhibition tests when C57BL/6 spleen cells were incubated simultaneously with ^{51}Cr -labeled AKR K36 target cells and varying numbers of nonlabeled cells demonstrated that the cytotoxic activity of normal C57BL/6 spleen cells was directed against an antigen(s) associated with several leukemias, but that was undetectable on normal thymocytes. Pretreatment of C57BL/6 spleen cells with carbonyl iron and a magnet, which removed phagocytic macrophages, did not decrease the cytotoxic activity for AKR K36 cells.

Cell-mediated immunity against tumor-associated antigens has been shown to be responsible for the rejection *in vivo* and killing *in vitro* of a variety of tumor and leukemia cells induced by chemical carcinogens and viruses in several species (for review, see ref. 1). Thymus-dependent, cell-mediated immunity has been demonstrated in rats immunized against a syngeneic leukemia induced by Gross virus (2) and in mice immunized against viral and chemical induced syngeneic leukemias (3-5). Although antibodies to structural proteins of the vertically transmitted leukemia viruses (6-8) and to antigens associated with leukemia cells (9-11) occur *naturally* in normal mice of strains of both high and low leukemia incidence, it is not clear whether a *cell-mediated* immune response against antigens associated with spontaneous leukemias also is elicited naturally in normal mice.

Our study was undertaken to determine if lymphocytes of normal mice of strains differing in incidence of spontaneous leukemia were cytotoxic for AKR leukemia cells. Results presented here demonstrate that spleen cells from normal mice of some strains with a low incidence of spontaneous leukemia, but not spleen cells from normal mice of high leukemic strains, lyse AKR leukemia cells *in vitro* as measured by the ^{51}Cr release assay.

MATERIALS AND METHODS

Animals and Leukemias. Inbred mice used in these studies were purchased from the Jackson Laboratories (Bar Harbor, Me.), with the exception that BALB/C mice were purchased from the Charles River Co. (Millerton, N.Y.). W/Fu rats were bred at the McArdle Laboratory.

Abbreviation: i.p., intraperitoneally.

Leukemia AKR K36 originated as a spontaneous AKR thymoma and has been maintained by transplantation in ascites form. Leukemia EL4 was induced in a C57BL mouse with 9,10-dimethyl-1,2-benzanthracene (12) and has been maintained by transplantation in ascites form. Leukemia ERLD was a radiation-induced thymoma of C57BL/6 mice that had been maintained by transplantation of spleen cells. Leukemia NTD was from a thymoma induced in a W/Fu rat by neonatal inoculation of thymus cells from C58 mice, and this murine leukemia virus (MuLV)-induced rat leukemia was maintained by transplantation of ascites cells in weanling W/Fu rats.

The AKR K36, EL4, and NTD cells were adapted to growth *in vitro* in RPMI-1640 (Grand Island Biological Co., New York) medium supplemented with penicillin (200 units/ml), streptomycin (200 $\mu\text{g}/\text{ml}$), and 10% heat-inactivated fetal calf serum. R1, a tissue culture line from a spontaneous thymoma of C58 origin, was obtained from the Salk Institute and also was maintained in RPMI-1640. All leukemia cell lines were shown to be free of mycoplasma.

Immunizations. C57BL/6 mice were inoculated intraperitoneally (i.p.) with 2×10^7 AKR K36 cells. BALB/C mice were inoculated i.p. with 1.5×10^7 spleen cells from a W/Fu rat.

Detection of Cytotoxic Spleen Cells by the ^{51}Cr Release Assay. Cell-mediated lysis was measured by the *in vitro* ^{51}Cr release assay (13). Leukemia AKR K36 was chosen as a standard target cell since it was derived from a *spontaneous* leukemia that has been characterized with respect to the expression of viral associated antigens (14), AKR K36 cells could be maintained in culture, and these cells were sensitive to lysis by allogeneic immune spleen cells in the ^{51}Cr -release assay.

(a) **Labeling of target cells:** *In vitro* adapted AKR K36 or NTD cells (3 to 8×10^6 in a volume of 0.7 ml) were labeled with 0.25 mCi of ^{51}Cr ($\text{Na}_2^{51}\text{CrO}_4$; 5 mCi/ml; New England Nuclear) for 1 hr at 37° and were washed three times with cold RPMI-1640 containing 15% fetal calf serum.

(b) **Preparation of effector spleen cells:** Spleen cell suspensions were prepared with ice-cold phosphate-buffered saline. The erythrocytes were lysed by a 2- to 3-sec exposure to distilled water. After the cells were washed, they were resuspended in RPMI-1640 with 10% fetal calf serum. Viability of the spleen cells ranged from 95 to 99%, as judged by exclusion of trypan blue stain.

(c) **Cytotoxic reaction:** The ^{51}Cr release assay was performed in plastic Linbro trays (IS-FB-96-TC, Linbro Chemical Co., Connecticut). To each of five or six replicate wells was added (a) spleen cells (2.5×10^6) in a volume of 0.1 ml, and (b) labeled target cells (10^4) in a volume of 0.1 ml, resulting in a spleen cell to target cell ratio of 250:1. The trays

were incubated at 37° in a humid atmosphere containing 5% CO₂ for 38–42 hr followed by the addition of 0.1 ml of cold medium to each well. The trays were then centrifuged at 400 × *g* for 8 min at 4°. A constant aliquot of the supernatant fluid was aspirated from each well, and the amount of radioactivity present in the supernatant fluid was determined in a gamma-counter. The maximum potential release of ⁵¹Cr from 1 × 10⁴ target cells was determined by freeze-thawing in distilled water. The spontaneous release of ⁵¹Cr was that released from 1 × 10⁴ labeled target cells incubated in medium alone after 38–42 hr and varied from 35 to 49% of the maximum release counts. The percentage of ⁵¹Cr released by spleen cells of various strains of mice (referred to as % specific ⁵¹Cr release) was calculated from the formula:

$$\frac{(X)\text{cpm} - (\text{spontaneous release})\text{cpm}}{(\text{maximal release})\text{cpm} - (\text{spontaneous release})\text{cpm}} \times 100$$

where X was the cpm released in the presence of spleen cells.

Competitive Inhibition of Target Cell Lysis by the Addition of Nonlabeled Cells. The assay for cell-mediated ⁵¹Cr release was performed as above with the exception that spleen cells were incubated in wells with nonlabeled thymocytes or leukemia cells and labeled target cells. Each reaction mixture had a final volume of 0.2 ml, consisting of 0.1 ml (2.5 × 10⁶) of spleen cells, 0.05 ml of competing cells, and 0.05 ml (1 × 10⁴) of labeled target cells.

Depletion of Macrophages from C57BL/6 Spleen Cell Suspensions. Spleen cell suspensions were incubated with carbonyl iron for 1 hr at 37°, by the method of Golstein *et al.* (15). Cells containing iron and cells adhering to iron clumps were then removed with a magnet. The cells were

Table 1. ⁵¹Cr release from AKR K36 leukemia cells mediated by spleen cells of normal mice of different strains.

Source of spleen cells	⁵¹ Cr released (mean cp5m ± SD)	% ⁵¹ Cr released*
AKR	2458 ± 42 (N.S.) [†]	-1.6
C57BL/6	2725 ± 91 (P < 0.001)	8.4
C57L	2790 ± 92 (P < 0.005)	10.8

* The % ⁵¹Cr released was based on a spontaneous release of 2501 ± 89 and a maximum release of 5180 ± 50.

† The numbers in parentheses refer to the probability (P) that the ⁵¹Cr released in the presence of spleen cells was significantly different from that released spontaneously by chance alone; this was calculated from the two-sample *t* test. N.S. = not significant (P > 0.05). cp5m, counts per 5 min.

washed once with medium and used in the ⁵¹Cr release assay.

RESULTS

Cytotoxicity of Normal Mouse Spleen Cells for Leukemia AKR K36. Spleen cells of 2- to 3-month-old normal mice of several strains were tested for cytotoxic activity against ⁵¹Cr-labeled AKR K36 cells. Preliminary experiments showed that after a 24-hr incubation the amount of ⁵¹Cr released by normal spleen cells was often not significantly above that released spontaneously from AKR K36 cells; however, by 38 hr in culture the spleen cells of some mice had mediated specific ⁵¹Cr release from AKR K36 cells.

The results of one of these experiments are shown in Table 1. In this experiment, incubation of labeled AKR K36 cells with spleen cells of normal mice of the C57L and

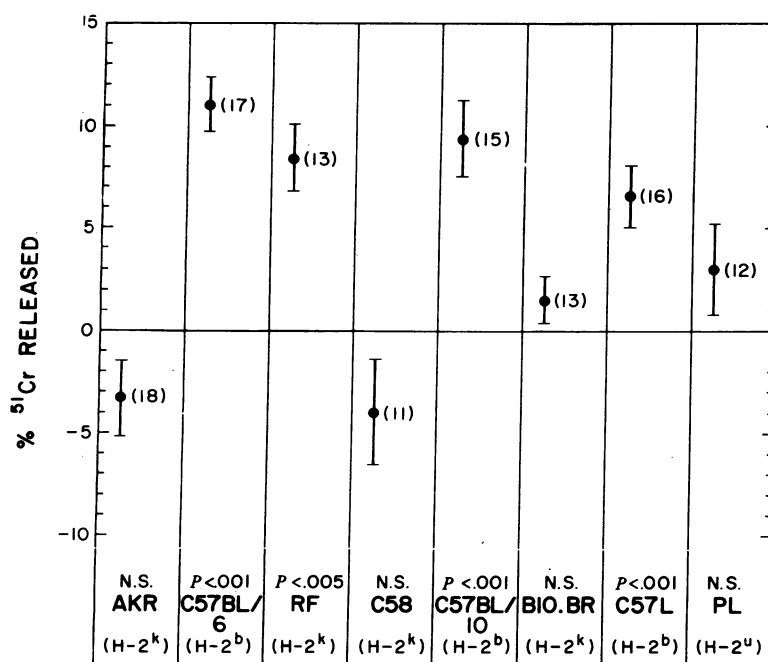


FIG. 1. ⁵¹Cr released from AKR K36 leukemia cells mediated by spleen cells of normal 2- to 3-month-old mice of different strains. Each spleen cell suspension was prepared from a pool of one to three mice and was incubated with labeled AKR K36 cells at a ratio of 250:1 for 38–42 hr. The bracketed bar represents the mean and standard error obtained for the percent ⁵¹Cr released by spleen cells of each strain. The numbers in parentheses are the number of experiments upon which the mean values were calculated. The P values refer to the probability that the percent ⁵¹Cr released from AKR K36 cells in the presence of spleen cells differed significantly from that released spontaneously (referred to as 0% in this figure) by chance alone.

C57BL/6 strains resulted in specific ^{51}Cr release of 10.8% and 8.4%, respectively. In contrast, incubation of AKR K36 cells with spleen cells from normal AKR mice did not result in specific ^{51}Cr release.

The results of 27 experiments, where spleen cells of normal mice from different strains were tested for cytotoxic activity against AKR K36 cells, are shown in Fig. 1. Spleen cells from mice of the C57BL/6, C57BL/10, RF, and C57L strains caused significantly more ^{51}Cr release from AKR K36 cells than that spontaneously released. The mean values \pm SE (based on 13 to 17 observations each) for the percent specific ^{51}Cr released from AKR K36 cells after incubation with spleen cell suspensions from mice of low leukemic strains were as follows: C57BL/6, $11 \pm 1.3\%$; C57L, $6.6 \pm 1.6\%$; RF, $8.4 \pm 1.7\%$; and C57BL/10, $9.3 \pm 2.0\%$.

Incubation of ^{51}Cr -labeled AKR K36 cells with spleen cells from B10.BR mice (a low leukemic strain) or PL mice (a high leukemic strain) did not result in significantly more ^{51}Cr release than that released spontaneously.

In contrast to the findings that spleen cells from normal mice of some low leukemic strains (C57BL/6, C57BL/10, RF, and C57L) were generally cytotoxic for AKR K36 cells, spleen cells from young AKR and C58 mice did not lyse AKR K36 cells. In fact, in most experiments, the amount of ^{51}Cr released in the presence of spleen cells from AKR and C58 mice was below that released spontaneously. A total of 18 separate spleen cell suspensions from AKR mice were tested against AKR K36 cells, with the mean value \pm SE for specific ^{51}Cr released being $-3.2 \pm 1.9\%$. The mean value \pm SE for specific ^{51}Cr released by 11 different C58 spleen cell suspensions was $-4.0 \pm 2.6\%$.

Competitive Inhibition of Target Cell Lysis by the Addition of Nonlabeled Cells. Further analysis of the cell-mediated cytotoxicity of AKR K36 cells was performed with spleen cells of C57BL/6 mice, a strain in which a consistent cytotoxic reaction was observed. Competitive-inhibition tests were performed to determine whether the antigen(s) associated with AKR K36 cells (that was recognized by C57BL/6 spleen cells) was (i) a histocompatibility antigen, (ii) an antigen of normal thymus cells, or (iii) a leukemia-associated antigen that was common to different leukemias. For this purpose, nonlabeled viable cells (leukemic or normal thymus of mice from H-2^k and H-2^b strains) were added in varying concentrations to a standard mixture (250:1) of C57BL/6 spleen cells and ^{51}Cr -labeled AKR K36 cells.

Results of three of these experiments are depicted in Fig. 2. As expected, the addition of nonlabeled AKR K36 cells to the reaction mixture was found to reduce the amount of cell-mediated cytotoxicity for ^{51}Cr -labeled AKR K36 cells to background levels. Inhibition of cell-mediated cytotoxicity also was observed when other nonlabeled leukemia cells were incubated with the C57BL/6 effector spleen cells and ^{51}Cr -labeled AKR K36 target cells. Results in Fig. 2 show that ERLD and EL4 leukemia cells (syngeneic with the effector C57BL/6 spleen cells) caused a dose-dependent inhibition of cell-mediated ^{51}Cr release from AKR K36 cells; thus, the antigen on AKR K36 cells against which C57BL/6 spleen cells were directed was not a normal histocompatibility antigen. The R1 cells, derived from a C58 spontaneous leukemia, also completely inhibited cytotoxicity of AKR K36 cells.

In contrast, the addition of nonlabeled thymus cells from AKR or C57BL/6 mice caused only a small reduction in ^{51}Cr released from AKR K36 cells in the presence of C57BL/6 spleen cells, and then only when high numbers of

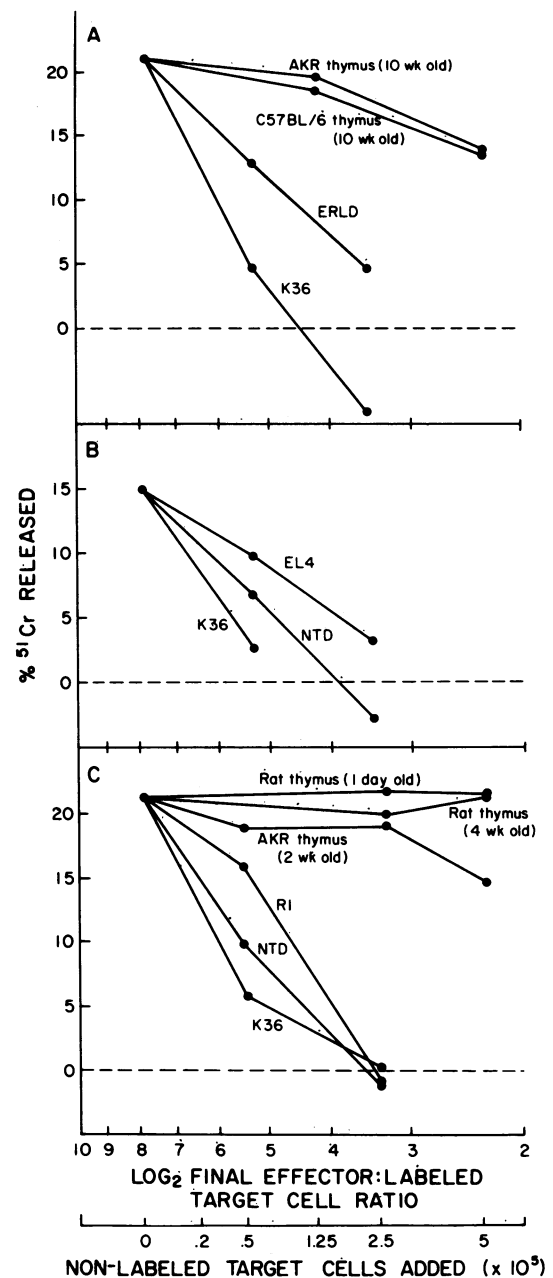


FIG. 2. Inhibitory effect of the addition of nonlabeled cells on ^{51}Cr release from AKR K36 cells mediated by spleen cells from C57BL/6 mice. Spleen cells from normal C57BL/6 mice were incubated with ^{51}Cr -labeled AKR K36 cells (at a ratio of 250:1) and with varying numbers of nonlabeled leukemia or normal thymus cells. The origins of the unlabeled leukemia cells are as follows: ERLD, radiation-induced leukemia of C57BL/6 origin; EL4, chemical carcinogen-induced leukemia of C57BL origin; R1, spontaneous leukemia of C58 origin; NTD, Gross virus-induced leukemia of W/Fu rat origin.

cells (5×10^5) were added to the incubation mixture. The slight reduction in cytotoxicity caused by the addition of high numbers of nonlabeled mouse thymus cells to the incubation mixture was probably due to the protective effect that these cells had on the spontaneous release of ^{51}Cr from AKR K36 cells. Although the addition of low numbers of thymus cells (1×10^5) did not inhibit the normal spleen cell-mediated lysis of AKR K36 cells, the same number of AKR thymus cells did competitively inhibit the lysis of AKR K36

by spleen cells of C57BL/6 mice that were immunized against AKR histocompatibility antigens (data not shown).

Also shown in Fig. 2 are two experiments demonstrating that the addition of nonlabeled W/Fu rat leukemia cells, NTD, completely inhibited the C57BL/6 spleen cell-mediated ^{51}Cr release from AKR K36 cells. In contrast, the addition of normal W/Fu thymus cells from newborn or 4-week-old rats caused no reduction in the amount of ^{51}Cr released.

The reduction of ^{51}Cr release from AKR K36 cells resulting from the addition of nonlabeled leukemia cells to the reaction mixture was due to antigenic competition rather than cell crowding or an inhibitory effect of leukemia cells on the cytolytic function of immune lymphocytes. This was demonstrated by the finding that the same leukemia cells that blocked normal C57BL/6 mouse spleen cells from lysing AKR K36 cells (Fig. 2) did not inhibit spleen cells from BALB/C mice immunized with rat spleen cells from lysing rat NTD leukemia cells (Fig. 3). Inhibition of ^{51}Cr release was observed only when nonlabeled NTD cells were added to the mixture of immune effector spleen cells and ^{51}Cr -labeled NTD cells.

Effect of Depletion of Macrophages on the Cytotoxicity of C57BL/6 Spleen Cells for AKR K36 Cells. Pretreatment of spleen cells from C57BL/6 mice with carbonyl iron, followed by the removal of phagocytic cells with a magnet, did not reduce the cytotoxic activity of the normal cells for AKR K36 cells; nor did this treatment reduce the cytotoxicity mediated by spleen cells from allogeneically immunized mice (Table 2). Instead, the depletion of macrophages resulted in an increased amount of ^{51}Cr released from the target cells. It was concluded that macrophages were not responsible for the normal spleen cell-mediated lysis of AKR leukemia cells and that the effector cells were of lymphocytic origin.

DISCUSSION

The results of experiments presented here demonstrate that spleen cells from 2- to 3-month-old normal mice of some strains lyse AKR leukemia cells *in vitro*. Incubation of ^{51}Cr -labeled AKR K36 cells with spleen cells from mice of some low leukemic strains (C57BL/6, C57BL/10, RF, and C57L) resulted in low but significant amounts of ^{51}Cr released as compared to that released in the presence of medium alone. In contrast, incubation of labeled AKR K36 cells with spleen cells from mice of two strains with high incidences of leukemia, C58 and AKR, resulted in less ^{51}Cr release than that released spontaneously.

Recently Gomard *et al.* (16) reported that spleen cells from 4- to 5-month-old (but not 2- to 3-month-old) AKR mice were cytotoxic for leukemia cells *in vitro*. In contrast to their findings, we have not demonstrated spleen cells from either young or old AKR mice that were cytotoxic for AKR K36 cells. Furthermore, in the study of Gomard *et al.* (16) phagocytic cells, probably macrophages, were responsible for the observed cytotoxicity. In our system pretreatment of "killer" spleen cells with carbonyl iron and a magnet did not reduce the cytotoxic activity of C57BL/6 spleen cells for AKR K36. In fact, the amount of ^{51}Cr released was increased, probably due in part to enrichment for the cytotoxic cells. It is also possible that the cells removed by the carbonyl iron-magnet treatment inhibited cell-mediated cytotoxicity by competing for the leukemia-associated antigens. A similar increase in cytotoxicity was observed in the EL4 system where spleen cells of mice immunized with syngeneic EL4 cells were treated with carbonyl iron and a magnet (4).

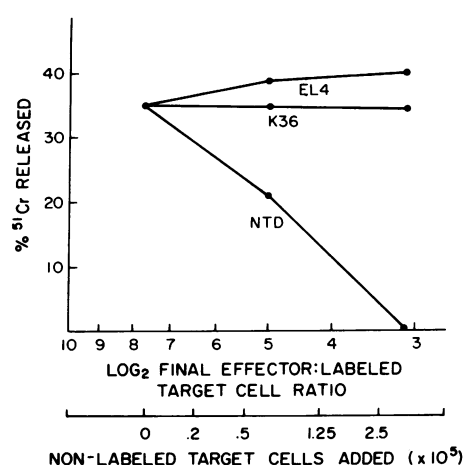


FIG. 3. Inhibitory effect of nonlabeled leukemia cells on ^{51}Cr release from the rat leukemia NTD mediated by spleen cells from BALB/C mice immunized with rat lymphocytes. Spleen cells from BALB/C mice immunized with rat lymphocytes were incubated with ^{51}Cr -labeled rat NTD cells at a ratio of 200:1. To this mixture were added varying numbers of nonlabeled leukemia cells. Competitive inhibition was observed only when the homologous NTD cells were added to the reaction mixture. This experiment was performed at the same time as the experiment illustrated in the middle panel of Fig. 2; nonlabeled leukemia cells used in both experiments were derived from a single pool.

Of particular interest in our study was the finding that spleen cells from C57BL/10 mice were invariably more cytotoxic for AKR K36 cells than were spleen cells from congenically derived B10.BR(H-2^k) mice. This suggests the possibility that H-2 linked immune response genes may play a role in controlling the generation of cytotoxic cells directed against leukemia-associated antigens. Cytotoxicity of AKR K36 cells by RF(H-2^k) spleen cells might be due to different immune response genes (within the H-2 region of the chromosome) from those of B10.BR, or it might indicate the influence of non-H-2 associated genes on the generation of cytotoxic cells.

The observation that two leukemias of C57BL/6 origin, EL4 and ERLD, inhibited C57BL/6 spleen cells from lysing AKR leukemia cells indicated that the cytotoxicity was not due to an immune reaction against normal histocompatibility antigens. Specificity of the competitive inhibition of lysis of AKR K36 cells by the addition of nonlabeled leukemia cells

Table 2. Effect of pretreatment of C57BL/6 spleen cells with carbonyl iron and a magnet on their cytotoxic activity against AKR K36 cells

Source of C57BL/6 spleen cells [†]	% ^{51}Cr release* in the presence of spleen cells treated by:	
	Medium alone	Carbonyl iron and magnet
A. Normal mice	9.0% (2662 ± 83)	19.3% (2927 ± 191)
B. Immunized with AKR spleen cells	75.3% (4383 ± 98)	93.0% (4827 ± 230)

* The % ^{51}Cr released was based on the spontaneous release (SR) and maximum release (MR) values (mean of 5 or 6 replicates ± SD) of: SR = 2496 ± 131, MR = 4993 ± 98. Values in parentheses are mean counts/5 min ± SD.

† The ratio of normal spleen cells to target cells was 250:1, the ratio of immune spleen cells to target cells was 200:1.

was established with the demonstration that EL4 cells did not inhibit cell-mediated ^{51}Cr release in an antigenically unrelated system. Furthermore, the addition of nonlabeled AKR thymus cells did not cause a greater reduction in C57BL/6 spleen cell-mediated ^{51}Cr release from AKR K36 cells than did the addition of C57BL/6 thymus cells. The slight reduction in ^{51}Cr release detected in the presence of high numbers of thymocytes was probably due to the protective effect of mouse thymocytes on the spontaneous release of ^{51}Cr , since normal mouse thymocytes (but not leukemia cells or rat thymocytes) were found to protect labeled AKR K36 cells from releasing ^{51}Cr spontaneously in the absence of effector cells. Thus, it was concluded that the antigen(s) of AKR K36 cells that was recognized by normal C57BL/6 mice was present on several different leukemia cells, but was not detected on normal mouse or rat thymus cells.

It is possible that the antigen detected on the cells of several leukemias is a gene product of the vertically transmitted leukemia viruses (which are present in all mouse cells) that is expressed after transformation of the lymphocytes to malignancy. These genes also would be expressed in rat thymocytes after transformation by murine leukemia virus. Alternatively, the common antigen(s) detected in this assay may be an embryonic antigen(s) which is re-expressed after leukemia transformation of cells (11) or it may be a common cell surface antigen, unrelated to viral structural proteins or fetal antigens, that is expressed after malignant transformation of thymocytes. Along this line, it should be noted that we have not determined whether this antigen is restricted exclusively to leukemia cells; it may have a tissue distribution wider than that determined in this study.

In summary, results reported here demonstrate that spleen cells from normal mice of some strains that have a low incidence of spontaneous leukemia are cytotoxic for AKR leukemia cells *in vitro*. It is of interest to note that mice of two of these strains (RF and C57BL/6) develop a high incidence of leukemia after exposure to irradiation. In this case, radiation results in bone marrow injury, immunosuppression, and release of endogenous leukemia virus (for review, see ref. 18). Possibly even without overt exposure to radiation or chemical carcinogens, lymphocytes in mice of low leukemic strains undergo transformation periodically. Such cells would then express antigens against which an effective cell-mediated immune response could be elicited. Our demonstration that lymphocytes obtained from normal mice of these strains are cytotoxic for leukemia cells supports this hypothesis. Although antibodies to endogenous leukemia viruses (6-8) and antibodies cytotoxic for leukemia cells have been found in mice of several high and low leukemia strains of mice (Nowinski, in preparation), it may be of significance that anti-leukemic "killer" cells were detected only in mice of low leukemic strains.

During the preparation of this manuscript we became aware that Dr. R. Kiessling and his colleagues (19, 20) have found that spleen cells of young normal mice of some genotypes lyse Moloney leukemia cells (YAC) *in vitro*. Similar to results reported here, they have found that spleen cells from C57BL/6 and C57L mice killed YAC cells, and that removal of macrophages from normal spleen cell suspensions did not decrease the cytotoxic activity. It remains to be determined whether the antigens expressed on AKR spontaneous leukemias and Moloney virus-induced leukemias against which normal spleen cells are directed are the same.

We thank Karla Dunn, Marianne Haney, Sheelah Anderson, Jane King, and Dr. Bryan Joiner for assistance and Paul Sondel for helpful discussions. This investigation was supported by Contract no. 1-CP-53513 from the National Cancer Institute, National Institute of Health, and Grant 11576 from the National Institute of Allergy and Infectious Diseases, National Institute of Health. This is Paper no. 36 from the Immunobiology Research Center and Paper no. 1818 from the Laboratory of Genetics.

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