# LEUKEMIA IN AKR MICE

## I. Effects of Leukemic Cells on Antibody-Forming Potential of Syngeneic and Allogeneic Normal Cells*

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Animals often succumb to syngeneic spontaneous or passaged tumors even though these tumors generally bear tumor-specific antigens to which the animals should respond. This is probably due to the fact that tumor-bearing animals are frequently immunosuppressed. A variety of mechanisms appear to be involved in this tumor-associated anergy. Serum blocking factors consisting of antigens, antibodies, or antigen-antibody complexes which prevent functioning of tumor reactive lymphocytes are found in a variety of viral and carcinogen-induced tumor systems (1–5). Immunosuppressive substances have been found in sera and ascitic fluid of mice bearing plasmacytomas, Ehrlich ascites tumors, and mammary adenocarcinomas (6–8). In vitro studies have shown that cell-free extracts or soluble factors released from mastocytomas, methylcholanthrene-induced tumors, fibrosarcomas, and several human tumors are immunosuppressive (9–12). Free virus has been implicated as being directly immunosuppressive in infections with Friend virus and radiation-induced leukemia virus (13–15). Direct tumor cell contact with immunocompetent lymphocytes appears to be required for immunosuppression in one methylcholanthrene-induced tumor system (16). Cells capable of inhibiting in vitro phytohemagglutinin (PHA)[1] lymphocyte stimulation are found in spleens of Moloney sarcoma tumor-bearing mice (17, 18). Autoimmunity in some tumor-bearing mice may be responsible for deletion of specific tumor-reactive lymphocytes (19).

Immune failure resulting from the onset of leukemia in AKR mice and the relationship of this immune failure to the leukemia transforming event has been discussed for many years (20–22). A generalized failure of the AKR immune surveillance mechanism does not appear to be the cause, but rather the consequence, of the onset of overt leukemia in these mice (Panfili and Golub, to be published; 23). Other workers have shown altered mitogeneic responsiveness of leukemic AKR cells to concanavalin A (Con A) (24) and PHA (24, 25), altered migration patterns and expression of theta antigen on leukemic thymocytes (25), and altered uptake and distribution of antigen in the leukemic spleen (26), but the relevance of these altered conditions to the cause of failure of the immune system of leukemic AKR mice is not clear.

We report here in vitro studies on the responsiveness of leukemic AKR cells to antigen and the effects of these leukemic cells on the immune responses of

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[1] *Abbreviations used in this paper:* LPS, lipopolysaccharide; PHA, phytohemagglutinin; PFC, plaque-forming cells; SRBC, sheep red blood cells; TNP-HRBC, trinitrophenyl horse red blood cell; TNP-LPS, trinitrophenyl lipopolysaccharide.

normal syngeneic and allogeneic lymphocyte populations. Leukemic AKR cells are capable of suppressing in vitro antibody responses of normal AKR lymphocytes as well as AKR cell-mediated responses to DBA mastocytomas (Panfili and Golub, to be published). We have chosen in vitro antibody production as a model system for investigating the suppressive mechanism of leukemic AKR cells. Our in vitro analysis of suppression by leukemic AKR cells suggests that the mechanism of immunosuppression in leukemic AKR mice is by direct cell contact, requires the tumor and normal cells to be syngeneic or semiallogeneic, and is overcome by an allogeneic effect.

## Materials and Methods

*Mice.* AKR/J mice, 5–8 wk old, as well as retired breeders, were obtained from The Jackson Laboratories, Bar Harbor, Maine. CBA, C57BL/6, C57BL/10 (B10), B10.A, B10.BR, 129, $C_3H/Fe$, AKR.M, C58, DBA/2, [C57BL/6 × DBA/2]$F_1$ ($BDF_1$), [AKR × C57BL/10]$F_1$ (AKB10), and [AKR × DBA/2 $F_1$] ($AKD2F_1$) were bred in our animal facilities using stocks obtained from The Jackson Laboratories. AKR.*H-2*$^b$ mice were the generous gift of Dr. Edward Boyse, Sloan-Kettering Institute, and the C57BL/6.AKR were the generous gift of Dr. Frank Lilly, Albert Einstein College of Medicine. Mice were housed five to a cage and maintained on high chlorine, low pH water.

AKR/J mice less than 4-mo old were used as a source of normal AKR cells, although older nonleukemic AKR mice gave normal responses. Overtly leukemic mice were characterized by their massively enlarged thymuses, spleens, lymph nodes, and livers.

*In Vitro Cultures.* In vitro immune responses were performed by the method of Mishell and Dutton (27). Lymphoid cells were cultured in 35-mm Falcon petri dishes (Falcon Plastics, Oxnard, Calif.) or, for cell contact experiments, in Karush type modified equilibrium dialysis chambers (Bellco Glass Inc., Vineland, N. J.) separated by 0.2 $\mu$m Nuclepore filters (General Electric Co., Plesanton, Calif.) (28).

*Antigens and Assays.* Sheep red blood cells (SRBC) were obtained from Colorado Serum Co., Denver, Colo. and were used as immunogen at $10^7$ cells per culture. Trinitrophenyl lipopolysaccharide (TNP-LPS) was prepared from trinitrobenzene sulfonic acid and lipopolysaccharide (LPS) by the method of Jacobs and Morrison (29); 0.1 $\mu$g of TNP-LPS were added per culture. Plaque assays were performed by the Cunningham modification of the Jerne plaque assay (30). Target cells consisted of SRBC or lightly-conjugated trinitrophenyl horse red blood cells (TNP-HRBC) prepared by the method of Rittenberg and Pratt (31).

*Irradiation of Cells.* Leukemic or normal cells were suspended to $10^7$ cells/ml in Hank's balanced salt solution (Grand Island Biological Co., Berkeley, Calif.) plus 5% fetal calf serum (Grand Island Biological Co.) and X-irradiated at doses indicated in the text. The source was a GE Maxitron 300 machine (General Electric, Milwaukee, Wis.) operated at 250 kV and 15 mA with 0.25 cm Cu and 1-mm Al filtration at a head height of 51 cm (34 cm for 8,000 R). A dose of 50 R/min (160 R/min for 8,000 R) was delivered over a uniform area, as determined dosimetrically with a Victoreen dosimeter (Victoreen Instrument Div., VLN Corp., Cleveland, Ohio). Irradiated cells were washed in Hank's and counted before use.

*Freezing Leukemic Cells.* Spleens, livers, and lymph nodes from overtly leukemic mice were dispersed in Hank's balanced salt solution (Grand Island Biological Co.) plus 5% fetal calf serum (Grand Island Biological Co.). Cells were suspended to $2 \times 10^7$ per ml in Dulbecco's Modified Eagle's Medium (Grand Island Biological Co.) plus 5% fetal calf serum and 8% dimethyl sulfoxide (Baker-reagent grade), and sealed in vials in 1-ml aliquots. The vials were frozen in a −70°C freezer and then placed in liquid $N_2$. Cells were thawed in a 37°C water bath and washed three times in buffer before use.

## Results

*Immune Unresponsiveness and Suppressive Effect of Leukemic AKR Cells.* Spleen cells from overtly leukemic AKR mice do not respond to the

thymus-dependent antigen SRBC and show a greatly reduced response to the thymus-independent antigen TNP-LPS in the in vitro Mishell-Dutton culture system (Table I). When normal AKR spleen cells are mixed with equal numbers of leukemic spleen or thymus cells, their ability to respond to SRBC is nearly abolished, and their ability to respond to TNP-LPS is reduced (Table I). Cells from the enlarged lymph nodes and livers of leukemic mice are also capable of suppressing the plaque-forming cells (PFC) responses of normal spleen cells (Table I).

To establish the relationship between the degree of suppression and the proportion of leukemic cells added per culture, varying numbers of leukemic cells were added to a constant number ($10^7$) of normal cells (Fig. 1). The degree of suppression observed varied with cells from different individual leukemic mice; 50% leukemic cells were usually required to reduce normal SRBC responses to less than 20% of control values, while 10% leukemic cells reduced responses to from 20 to 80% of control value. The variation in suppressive ability of cells from different mice may be a function of the proportion of leukemic cells present in the organ being used as a source of leukemic cells, and thus the degree of metastatis of the leukemia, or may reflect inherent differences in suppressive ability of different leukemic cells. Leukemic cells which have been stored frozen in liquid nitrogen maintain full suppressive ability, thus allowing repeated examination of cells from individual mice (Table I). The thymus-independent response of normal spleen cells to TNP-LPS was always more resistant to suppression by leukemic cells than the thymus-dependent response to SRBC (Fig. 1); however, this is probably because LPS is capable of overcoming immuno-

TABLE I

*Suppression of Responses of Normal AKR Spleen Cultures by Leukemic AKR Thymus, Spleen, Lymph Node, and Liver Cells**

| Exp no. | Cells | SRBC PFC/culture | % control | TNP-LPS PFC/culture | % control‡ |
|---|---|---|---|---|---|
| 1 | Normal spleen | 278 ± 74 | | 205 ± 43 | |
| | Leukemic spleen | 0 | | 40 ± 25 | |
| | Normal spleen plus leukemic spleen | 0 | 0 | 50 ± 8 | 24 |
| 2 | Normal spleen | 1,260 ± 291 | | 1,253 ± 81 | |
| | Leukemic thymus | 0 | | 35 ± 3 | |
| | Normal spleen plus leukemic thymus | 33 ± 21 | 3 | 615 ± 175 | 49 |
| 3 | Normal spleen | 2,731 ± 450 | | NT§ | |
| | Leukemic spleen | 0 | | NT | |
| | Normal spleen plus leukemic spleen | 58 ± 48 | 2 | NT | |
| | Normal spleen plus leukemic thymus | 313 ± 53 | 11 | NT | |
| | Normal spleen plus leukemic lymph node | 275 ± 50 | 10 | NT | |
| 4 | Normal spleen | 574 ± 9 | | NT | |
| | Leukemic spleen | 0 | | NT | |
| | Normal spleen plus leukemic spleen | 125 ± 18 | 22 | NT | |
| | Normal spleen plus leukemic thymus | 93 ± 27 | 16 | NT | |
| | Normal spleen plus leukemic liver | 55 ± 8 | 10 | NT | |

* $10^7$ normal AKR spleen cells or $10^7$ normal AKR spleen cells plus $10^7$ leukemic cells were added per culture. SRBC were added at $10^7$ cells per culture and TNP-LPS at 1 μg per culture. TNP-LPS cultures were assayed at day 3 and SRBC cultures at day 4.

‡ Percent control, PFC/culture of leukemic plus normal cells divided by PFC/culture of normal cells.

§ NT, not tested.

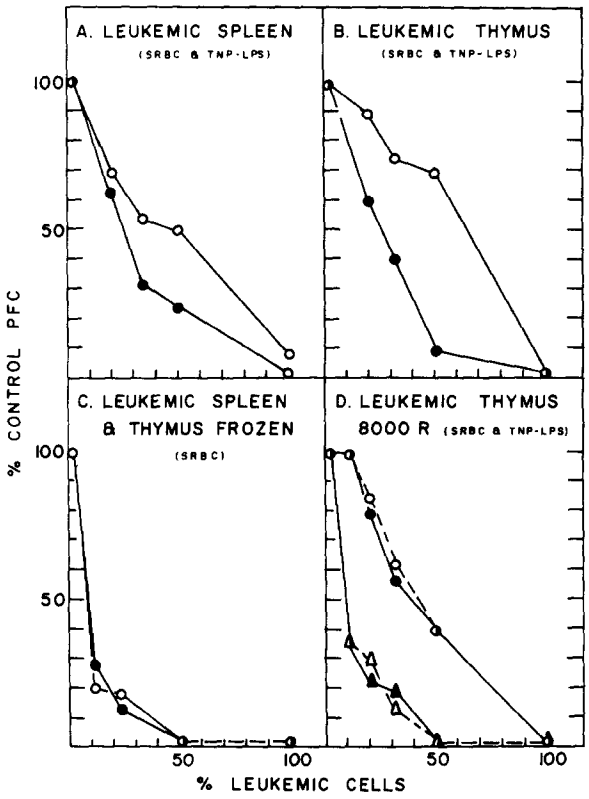


FIG. 1. Titration of suppressive activity of leukemia cells after freezing or irradiation. Varying numbers of leukemic cells ($10^7$, $5 \times 10^6$, $2.5 \times 10^6$, or $1.25 \times 10^6$) were added to $10^7$ normal AKR spleen cells plus SRBC or TNP-LPS (resulting in 50, 33, 20, and 12% leukemic cells per culture). Cultures containing $10^7$ normal cells alone or $10^7$ leukemic cells alone plus antigen served as controls. TNP-LPS cultures were assayed on day 3 and SRBC cultures on day 4. Values are expressed as percent of control PFC (i.e., PFC of $10^7$ normal spleen cells) per culture. Leukemic spleen and thymus cells in parts A and B were obtained from the same leukemic mouse and were used unfrozen (○ = TNP-LPS; ● = SRBC). Leukemic cells in part C were obtained from a different leukemic mouse and were used after freezing (○ = leukemic thymus; ● = leukemic spleen). Leukemic thymus cells in part D were from a third leukemic mouse and were used unfrozen (○ = 8,000 R, TNP-LPS; ● = unirradiated, TNP-LPS; △ = 8,000 R, SRBC; ▲ = unirradiated, SRBC).

suppression by leukemic cells (Roman and Golub, manuscript in preparation).

Leukemic cells obtained from mice bearing spontaneous leukemias did not replicate well in vitro, and thus the suppressive effect exerted by these cells could not be attributed to their simply having "overgrown" the cultures. As proof of this, leukemic cells which have been X-irradiated with 8,000 R still possess full suppressive ability (Fig. 1 D).

*Kinetics of Suppression.* To determine if development of the in vitro immune response was sensitive to inhibition by leukemic cells throughout the culture period, $10^7$ leukemic cells were added to cultures containing $10^7$ normal spleen cells plus SRBC at various days of culture. For consistency, frozen leukemic cells from the same mouse were used throughout the experiment. $10^7$

normal spleen cells without added leukemic cells served as controls. Leukemic cells were added on days 0, 1, 2, 3, or 4, and cultures were assayed on day five, which was the day of peak control response. The results (Fig. 2) expressed as percent of control value show that leukemic cells were able to arrest further development of PFC even when added at the 4th day of culture.

*Requirement for Cell Contact.* To establish whether soluble factors play a role in leukemic cell suppression of normal PFC response, leukemic cells or mixtures of leukemic and normal cells were cultured for 4 days in Mishell-Dutton culture conditions. Medium from these cultures was centrifuged and filtered through 0.45 $\mu$m filters or left unfiltered. 0.5 ml of the supernatant fluid was mixed with an equal volume of fresh medium and used to culture normal AKR spleen cells. The PFC responses of these cultures were not reduced, although the leukemic cells themselves, which had been in culture for 4 days, retained their immunosuppressive capabilities (Table II).

To further establish the failure of leukemic cells to elaborate soluble suppressive factors, leukemic and normal cells were cultured opposite one another in Karush type chambers separated by 0.2 $\mu$m Nuclepore filters as described by Calkins and Golub (28). In such chambers medium is free to flow from one cell population to another, but cell contact is prohibited. The data in Table III show that normal cells grown transfilter from leukemic cells are able to generate normal anti-SRBC responses. This suggests that cell contact is needed for suppression.

*Specificity of Suppression for Syngeneic Cells.* A requirement for cell contact between leukemic and normal cells could indicate that a specific recognition of cell surface moieties is necessary for suppression to occur. We therefore tested the ability of leukemic AKR cells to suppress a variety of allogeneic cells. Leukemic cells from 80% (24 of 30) of the leukemic AKR mice which we have tested for specificity have not been suppressive for allogeneic cells, as shown by the following experiments. Leukemic AKR cells are unable to suppress anti-

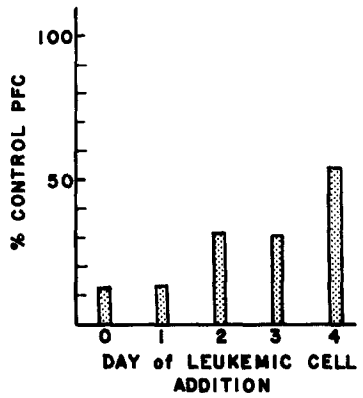


FIG. 2. Cultures of $10^7$ normal spleen cells were challenged with SRBC on day 0. On days 0 through 4, $10^7$ leukemic spleen cells were added to the cultures and cultures were assayed on day 5. Frozen leukemic cells from one leukemic mouse were used throughout. Cultures without added leukemic cells served as controls and had 512 ± 62 PFC on day 5, which was the day of peak response.

TABLE II
*Test for Suppression of Normal AKR Spleen Cell PFC Responses by Supernates of Leukemic or Leukemic plus Normal Cultures*

| Cells* | Supernate source‡ | Filtered | PFC/culture |
|---|---|---|---|
| Normal AKR | — | – | 440 ± 53 |
| Normal AKR | Leukemic spleen | – | 458 ± 31 |
| Normal AKR | Leukemic spleen | + | 482 ± 21 |
| Normak AKR | Leukemic plus normal | – | 463 ± 82 |
| Normal AKR | Leukemic plus normal | + | 453 ± 21 |

* $10^7$ normal AKR spleen cells plus SRBC were added to all cultures; PFC assays were performed on day 4.

‡ Cultures of leukemic spleen cells alone or mixtures of leukemic spleen cells plus normal AKR spleen cells were harvested at day 4 of culture, pelleted at 1,800 RPM for 30 min, and the supernates filtered through 0.45 μm filters or left unfiltered. Filtered or unfiltered supernates (0.5 ml) were added to $10^7$ normal AKR spleen cells plus SRBC. Control experiments indicated that the leukemic cells used for the generation of these supernates were capable of suppressing SRBC responses to 6% of control when used fresh and to 7% of control when used after 4 days in culture.

TABLE III
*Lack of Suppression of Normal AKR SRBC Response by Leukemic AKR Cells Across a Filter*

| Group | Chamber side 1* | Chamber side 2 |
|---|---|---|
| I | Normal AKR + SRBC<br>757 ± 106‡ | Normal AKR + SRBC<br>690 ± 36 |
| II | Normal AKR + SRBC<br>682 ± 232 | Normal AKR – SRBC<br>160 ± 57 |
| III | Normal AKR + SRBC<br>1,155 ± 337 | Leukemic AKR + SRBC<br>2 ± 2 |
| IV | Normal AKR + SRBC<br>795 ± 150 | Normal AKR + leukemic AKR + SRBC<br>170 ± 26 |

* Chambers were separated by 0.2 μm Nuclepore filters. Each side of the chamber contained $10^7$ normal AKR spleen cells with or without SRBC or $10^7$ leukemic spleen cells plus SRBC, or $10^7$ normal plus $10^7$ leukemic cells plus SRBC.

‡ Mean PFC per chamber ± the SE of the mean for four replicate chambers. PFC assays were performed on day 5.

SRBC responses of allogeneic cells which differ from AKR in both background and *H-2* genes, such as DBA/2, C57BL/6, $BDF_1$, 129, and B10 (Table IV). DBA/2 has the same *M* locus as AKR (*M-1*, 32), and the 129 expresses the $G_{IX}$ Gross virus antigen, as does AKR (33). Leukemic AKR cells are also unable to suppress allogeneic cells which differ from AKR in background, but have the same or part of the same *H-2* haplotype as AKR ($H\text{-}2^k$), such as C58, CBA, $C_3H$, C57BL/6.AKR, A, B10.A, and B10.BR (Table V). It is of interest that C58, which like AKR has a high incidence of leukemia, is not suppressed by leukemic

TABLE IV

*Failure of Leukemic AKR Cells to Suppress Allogeneic Cells with both Background and H-2 Differences**

| Exp no. | Strain | H-2 haplotype | Leukemic AKR cells added | PFC/culture | % control |
|---|---|---|---|---|---|
| 1 | AKR | kkkkkk | — | 557 ± 57 | |
| | | | Thymus | 150 ± 45 | 27 |
| | C57BL/6 | bbbbbb | — | 2,137 ± 345 | |
| | | | Thymus | 2,143 ± 700 | 100 |
| 2 | AKR | kkkkkk | — | 555 ± 120 | |
| | | | Thymus | 43 ± 8 | 8 |
| | B10 | bbbbbb | — | 1,530 ± 269 | |
| | | | Thymus | 6,615 ± 931 | >100 |
| 3 | | | — | 730 ± 204 | |
| | AKR | kkkkkk | Spleen | 15 ± 5 | 2 |
| | | | Thymus | 22 ± 13 | 3 |
| | $BDF_1$ | dddddd / bbbbbb | — | 725 ± 265 | |
| | | | Spleen | 1,598 ± 153 | >100 |
| | | | Thymus | 878 ± 96 | >100 |
| 4 | | | — | 273 ± 16 | |
| | AKR | kkkkkk | Spleen | 85 ± 18 | 31 |
| | | | Thymus | 50 ± 11 | 18 |
| | DBA/2 | dddddd | — | 1,034 ± 355 | |
| | | | Spleen | 2,190 ± 651 | >100 |
| | | | Thymus | 1,072 ± 424 | 100 |
| | C57BL/6 | bbbbbb | — | 5,557 ± 717 | |
| | | | Spleen | 6,794 ± 358 | >100 |
| | | | Thymus | 4,432 ± 287 | 80 |
| | $BDF_1$ | bbbbbb / dddddd | — | 2,902 ± 252 | |
| | | | Spleen | 6,524 ± 980 | >100 |
| | | | Thymus | 3,712 ± 334 | >100 |
| | B10 | bbbbbb | — | 1,072 ± 174 | |
| | | | Spleen | 4,365 ± 370 | >100 |
| | | | Thymus | 2,264 ± 513 | >100 |
| | 129 | bbbbbb | — | 929 ± 105 | |
| | | | Spleen | 1,469 ± 549 | >100 |
| | | | Thymus | 1,267 ± 223 | >100 |

* $10^7$ normal spleen cells of the strain indicated were cultured alone or with $10^7$ leukemic spleen or thymus cells plus SRBC. Fresh leukemic cells from different leukemic mice were used for each experiment shown except experiment 1 in which frozen leukemic cells were used. Cultures were assayed on day 4. Percent control refers to PFC of cultures with leukemic cells divided by PFC of cultures without leukemic cells × 100. $10^7$ leukemic spleen cells challenged with SRBC gave zero PFC/culture in all experiments.

AKR cells. Furthermore, leukemic AKR cells do not suppress AKR.*H-2*$^b$ or AKR.M cells with the same background as AKR but differing in *H-2* haplotype (Table VI). AKR.M cells differ from AKR cells only at the *D* region of *H-2* and the T/a locus (34).

Thus, it appears that neither background nor *H-2* similarities alone are sufficient for a cell to be suppressed by leukemic AKR cells, and that any genetic difference which can result in an allogeneic reaction by normal cells toward foreign determinants on leukemic cells is sufficient to prevent suppression from occurring. We therefore investigated suppression in a semiallogeneic system in which the normal cells are unable to respond allogeneically to leukemic cells. Under these conditions we found that the leukemic cells were able to suppress. This is shown in Table VII; the $F_1$s of AKR and two nonsuppressible strains (B10 and DBA/2) are suppressed by leukemic AKR cells.

*Effect of Allogeneic Cells on Syngeneic Suppression.* To determine whether an allogeneic reaction was sufficient to overcome suppression, small numbers of irradiated allogeneic cells were added to cultures of normal AKR spleen cells plus leukemic AKR cells. In this situation, the normal AKR cells are able to respond to determinants on the irradiated allogeneic cells. As shown in Table VIII, this allogeneic stimulation abrogates the suppressive ability of leukemic cells. In fact, addition of semiallogeneic cells overcomes suppression of AKR cells despite the fact that semiallogeneic cells are themselves suppressed by leukemic cells. The stimulation of PFC over background (i.e., AKR cells alone) caused by addition of irradiated semiallogeneic cells is, however, lower in the presence of leukemic cells (Table VIII).

## Discussion

The debate as to whether a state of immunological anergy is the cause or the effect of neoplasia has been a continuing one for many years (35, 36). Our data suggest that in the case of AKR leukemias, at least, the neoplastic cells themselves can induce a state of anergy in normally functioning lymphocytes. This leukemia-mediated suppression of cells which might otherwise respond against the tumor cells could have great significance in terms of the pathogenesis of the leukemia in AKR mice. We have investigated the suppression of a thymus-dependent antibody response here, but we have established that cell-mediated responses are suppressed by leukemic cells as well (Panfili and Golub, manuscript in preparation). The fact that such functionally diverse organs as thymus, spleen, lymph node, and liver contain immunosuppressive cells (Table I) argues that the widely metastasized leukemic cells are themselves suppressing. However, it cannot yet be ruled out that transformed cells turn on suppressive function in normal cell populations, and that these cells have (or assume) a heterogeneous organ distribution. The nature of the suppressive cell or cells in leukemic AKR mice is currently under investigation.

The thymus-independent TNP-LPS response of normal AKR spleen cells is more resistant to suppression by leukemic cells than the thymus-dependent SRBC response (Table I and Fig. 1). This probably does not reflect inherent differences in sensitivity to suppression of either T or B cells, but more likely reflects the ability of LPS to overcome suppression (Roman and Golub, to be

TABLE V

*Failure of Leukemic AKR Cells to Suppress Allogeneic Cells with Background Differences and H-2 Similarities**

| Exp no. | Strain | *H-2* haplotype | Leukemic AKR cells added | PFC/culture | % control |
|---|---|---|---|---|---|
| 1 | | | — | 878 ± 168 | |
| | AKR | kkkkkk | Spleen | 25 ± 22 | 3 |
| | | | Thymus | 63 ± 58 | 7 |
| | | | — | 724 ± 179 | |
| | C58 | kkkkkk | Spleen | 907 ± 298 | >100 |
| | | | Thymus | 1,230 ± 240 | >100 |
| 2 | AKR | kkkkkk | — | 2,731 ± 450 | |
| | | | Spleen | 58 ± 48 | 0 |
| | CBA | kkkkkk | — | 392 ± 128 | |
| | | | Spleen | 410 ± 51 | >100 |
| 3 | AKR | kkkkkk | — | 555 ± 120 | |
| | | | Thymus | 43 ± 8 | 8 |
| | CBA | kkkkkk | — | 120 ± 4 | |
| | | | Thymus | 152 ± 29 | 100 |
| | $C_3H$/Fe | kkkkkk | — | 167 ± 11 | |
| | | | Thymus | 207 ± 21 | >100 |
| | B10·A | kkkddd | — | 2,745 ± 589 | |
| | | | Thymus | 2,790 ± 153 | 100 |
| 4 | | | — | 362 ± 65 | |
| | AKR | kkkkkk | Spleen | 105 ± 19 | 29 |
| | | | Thymus | 12 ± 16 | 3 |
| | | | — | 848 ± 103 | |
| | CBA | kkkkkk | Spleen | 817 ± 60 | 96 |
| | | | Thymus | 776 ± 79 | 92 |
| | | | — | 160 ± 22 | |
| | $C_3H$/Fe | kkkkkk | Spleen | 315 ± 62 | >100 |
| | | | Thymus | 401 ± 5 | >100 |
| | | | — | 35 ± 8 | |
| | A | kkkddd | Spleen | 337 ± 56 | >100 |
| | | | Thymus | 218 ± 21 | >100 |
| | | | — | 510 ± 224 | |
| | B10.A | kkkddd | Spleen | 3,802 ± 587 | >100 |
| | | | Thymus | 742 ± 63 | >100 |

TABLE V—*Continued*

| Exp no. | Strain | *H-2* haplotype | Leukemic AKR cells added | PFC/culture | % control |
|---|---|---|---|---|---|
| | B10.BR | kkkkkk | — | 967 ± 88 | |
| | | | Spleen | 7,065 ± 670 | >100 |
| | | | Thymus | 4,287 ± 143 | >100 |
| | C57BL/6.AKR | kkkkkk | — | 539 ± 88 | |
| | | | Spleen | 4,049 ± 550 | >100 |
| | | | Thymus | 2,328 ± 620 | >100 |

* $10^7$ normal spleen cells of the strain indicated were cultured alone or with $10^7$ leukemic spleen or thymus cells plus SRBC. Fresh leukemic cells from different leukemic mice were used for each experiment shown. Cultures were assayed on day 4. Percent control refers to PFC of cultures with leukemic cells × 100. The C57BL/6.AKR mice were a generous gift of Dr. F. Lilly. $10^7$ leukemic spleen cells challenged with SRBC gave zero PFC/culture in all experiments.

TABLE VI

*Failure of Leukemic AKR Cells to Suppress Allogeneic Cells with AKR Background Similarities and H-2 Differences**

| Exp no. | Strain | H-2 haplotype | Leukemic AKR cells added | PFC/culture | % control |
|---|---|---|---|---|---|
| 1 | | | — | 611 ± 108 | |
| | AKR | kkkkkk | Spleen | 98 ± 29 | 16 |
| | | | Thymus | 117 ± 45 | 19 |
| | | | — | 200 ± 35 | |
| | AKR.M | kkkkkq | Spleen | 370 ± 97 | >100 |
| | | | Thymus | 252 ± 37 | >100 |
| | | | — | 645 ± 121 | |
| | AKR.*H-2*$^b$ | bbbbbb | Spleen | 990 ± 252 | >100 |
| | | | Thymus | 877 ± 96 | >100 |
| 2 | | | — | 255 ± 30 | |
| | AKR | kkkkkk | Spleen | 27 ± 3 | 11 |
| | | | Thymus | 41 ± 37 | 16 |
| | | | — | 362 ± 51 | |
| | AKR.*H-2*$^b$ | bbbbbb | Spleen | 720 ± 42 | >100 |
| | | | Thymus | 573 ± 79 | >100 |

* $10^7$ normal spleen cells of the strain indicated were cultured alone or with $10^7$ leukemic spleen or thymus cells plus SRBC. Fresh leukemic cells from two different leukemic mice were used for the two experiments shown. Cultures were assayed on day 4. Percent control refers to PFC of cultures with leukemic cells divided by PFC of cultures without leukemic cells × 100. The AKR.*H-2*$^b$ mice were a generous gift from Dr. Edward Boyse. $10^7$ leukemic spleen cells challenged with SRBC gave zero PFC/culture in all experiments.

TABLE VII
*Ability of Leukemic AKR Cells to Suppress Semiallogeneic Cells**

| Strain | H-2 haplotype | Leukemic AKR cells added | PFC/culture | % control |
|---|---|---|---|---|
| AKR | kkkkkk | — | 1,245 ± 246 | |
| | | Spleen | 10 ± 8 | 0 |
| | | Thymus | 40 ± 40 | 3 |
| B10 | bbbbbb | — | 5,115 ± 538 | |
| | | Spleen | 7,650 ± 420 | >100 |
| | | Thymus | 5,425 ± 962 | >100 |
| DBA/2 | dddddd | — | 802 ± 105 | |
| | | Spleen | 905 ± 81 | 100 |
| | | Thymus | 803 ± 18 | 100 |
| $AKD_2F_1$ | kkkkkk / dddddd | — | 458 ± 64 | |
| | | Spleen | 30 ± 5 | 6 |
| | | Thymus | 40 ± 21 | 9 |
| $AKB10F_1$ | kkkkkk / bbbbbb | — | 5,580 ± 562 | |
| | | Spleen | 472 ± 69 | 8 |
| | | Thymus | 593 ± 9 | 11 |

* $10^7$ normal spleen cells of the strain indicated were cultured alone or with $10^7$ fresh leukemic spleen cells plus SRBC. Cultures were assayed on day 5. Percent control refers to PFC of cultures with leukemic cells divided by PFC of cultures without leukemic cells × 100. $10^7$ leukemic spleen cells challenged with SRBC gave zero PFC/culture in all experiments.

published). The cell type(s) which are sensitive to suppression by leukemic AKR cells is presently being investigated. However, the kinetic experiments (Fig. 2) would indicate that, at least in this situation, addition of leukemic cells after initiation of clonal expansion of B cells halts further B-cell proliferation. Addition of leukemic cells at later times during the culture period did not completely abolish PFC, suggesting that leukemic cells are not suppressing by killing spleen cells.

The fact that leukemic AKR cells act as efficient immunosuppressants when added at any time during the 4-day culture period suggests a different mechanism of suppression than has been reported for other in vitro tumor suppressor systems in which tumor cells must be added within 24 h after initiation of culture in order to suppress (9, 15). Irradiated leukemic cells are fully suppressive (Table I), which also suggests a different mechanism of suppression than is found with Friend virus-infected cells where X-ray and mitomycin C abolish suppressive ability (15).

Leukemic AKR cells require contact with normal lymphocytes to suppress their immune responses (Tables II and III). The fact that suppressive factors are not elaborated by leukemic cells explains why relatively large numbers of cells are required for efficient suppression. In those in vitro tumor suppression

TABLE VIII
*An Allogeneic Effect Overcomes Suppression by Leukemic AKR Cells**

| Normal cells | Irradiated cells added‡ | Leukemic AKR cells added | PFC/culture | % control§ |
|---|---|---|---|---|
| AKR | — | — | 630 ± 114 | |
| AKR | — | Spleen‖ | 128 ± 46 | 20 |
| AKR | AKR‡ | — | 728 ± 143 | |
| AKR | AKR | Spleen | 68 ± 23 | 10 |
| AKR | DBA/2 | — | 3,305 ± 391 | |
| AKR | DBA/2 | Spleen | 5,332 ± 286 | >100 |
| AKR | $AKD_2F_1$ | — | 3,105 ± 109 | |
| AKR | $AKD_2F_1$ | Spleen | 1,845 ± 338 | >100 |
| DBA/2 | — | — | 2,250 ± 478 | |
| DBA/2 | — | Spleen | 2,588 ± 192 | 100 |
| $AKRD_2F_1$ | — | — | 1,343 ± 385 | |
| $AKRD_2F_1$ | — | Spleen | 385 ± 140 | 28 |

* Normal spleen cells of the strain indicated were cultured at $10^7$ cells per culture. $10^6$ X-irradiated cells (2,000 R) and/or $10^7$ frozen leukemic spleen cells were added where indicated. SRBC were added to all cultures and cultures were assayed on day 4.

‡ $10^7$ irradiated AKR, DBA/2, and $AKD_2F_1$ cells challenged with SRBC gave zero PFC on day 4 of cultures.

§ Percent control refers to percent of PFC response in the absence of irradiated cells or leukemic cells.

‖ $10^7$ leukemic spleen cells challenged with SRBC gave zero PFC/culture in all experiments.

systems which are mediated by soluble factors, a significant degree of suppression may be achieved with relatively few tumor cells (9, 11, 15).

This requirement for cell contact led us to explore the degree of surface antigenic homology required for suppression. Our data indicates that homology either within the *H-2* region or in background genes alone is not sufficient to permit suppression by leukemic AKR cells, since a difference in either *H-2* or background genes prevents suppression (Tables IV, V, and VI). The $F_1$s of AKR and two nonsuppressible strains (B10 and DBA/2) were, however, suppressed (Table VII). It is possible that two genetic loci are required for suppression, one within the *H-2* region and one outside. On the other hand, it may be that cell surface homology is not required for suppression at all, but rather that any cell capable of making an allogeneic response to the leukemic cell determinants or to any other foreign cell surface antigens is able to escape suppression. The fact that normal AKR cells are not suppressed by leukemic AKR cells when irradiated allogeneic cells are present argues that this may be the case (Table VIII). Bortin et al. reported a significant delay in onset of passaged AKR leukemia after injections of allogeneic cells, although these authors attributed this finding to a killing of leukemic cells by the allogeneic cells (37).

If the immunosuppressive activity of the leukemic cell is important in the pathogenesis of the AKR thymic leukemia and the suppression can be overcome by allogeneic interaction, it may be possible to alter the course of the disease by treatment with allogeneic cells. Experiments along this line are now in progress.

## Summary

Cells from the spleen, thymus, lymph node, and liver of leukemic AKR mice suppress in vitro antibody responses of normal syngeneic and semiallogeneic cells. This suppression can be mediated by irradiated leukemic cells, requires cell contact between leukemic and normal cells, and may occur at any time during the in vitro culture period. Leukemic AKR cells do not suppress antibody responses of allogeneic cells, even when allogeneic cells have *H-2* or background genes homologous with AKR. Leukemic cells do, however, suppress cells that are unable to respond allogeneically to leukemic AKR cells, such as cells of the $F_1$s of AKR. Suppression of normal AKR antibody responses by leukemic AKR cells may be overcome by addition of irradiated allogeneic cells. The fact that leukemic AKR cells are able to suppress normal lymphocyte responses may be of significance in pathogenesis of leukemia in these mice.

*Note Added in Proof.* The leukemic and normal AKR mice used in these experiments were female. We now have evidence that suppression across sex barriers is infrequent, probably due to recognition of male-female antigenic differences.

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