Defect in Cellular Immunity of Leukemia Virus-Infected Mice Assessed by a Macrophage Migration-Inhibition Assay[1] (35216)

HERMAN FRIEDMAN AND WALTER S. CEGLOWSKI[2]

Departments of Microbiology, Albert Einstein Medical Center and Temple University School of Medicine, Philadelphia, Pennsylvania 19141

It is now generally well recognized that immunologic inadequacy is closely associated with leukemogenesis. Impaired humoral and cellular immune capacity during virus-induced leukemogenesis in mice has been repeatedly demonstrated (1–9). It has been suggested that the leukemia virus-mediated immune suppression is a consequence of a competition between virus and antigen for a common precursor cell. Thus, even though the role of the immune system in development of virus-induced leukemia is equivocal, it is still important in the understanding of the mechanism of virus-mediated leukemogenesis.

In this regard, a number of studies have shown that leukemia viruses such as Friend, Rauscher, Maloney, and Gross result in a marked immunosuppression (1–9). Many of these studies were concerned with humoral antibody responses. Serum antibody titers are depressed in mice inoculated with leukemogenic viruses before either primary or secondary immunization. Furthermore, the number of specific antibody forming cells to antigens like sheep erythrocytes and *E. coli* lipopolysaccharide is also suppressed in leukemic mice (4, 5–10).

Tumor virus related effects on cellular immunity have been demonstrated only in the case of skin allografts. It was reported that mice infected at birth with Gross passage A virus had a moderate prolongation of graft survival time when transplanted with skin from donor mice differing at a non-H2 locus (11). Similar skin graft reactivity was found to be depressed in preleukemic AKR mice harboring the Gross virus (12). In the present study cellular immunity of mice infected with Friend leukemia virus was studied using a macrophage migration-inhibition assay to assess immunocomptence of mice to antigens of tubercle bacilli (13, 14). Both normal control and virus-infected mice were sensitized to mycobacterial antigens and cellular immunity was determined at various times by the *in vitro* machrophage migration assay.

Methods and Materials. Animals. BALB/c mice were obtained from Flow Research Animals, Dublin, Va. They were approximately 6–8 weeks of age and weighed 15–18 g at the start of each experiment.

Tumor virus. Friend leukemia virus (FLV) was used for this study as described previously (6, 8). The stock virus preparation, consisting of cell-free filtrates of infected spleens in 0.5 ml of sterile Hanks' salt solution, was injected intraperitoneally (ip) into test mice so that each animal received 10^3 ID_{50} units of virus. Control animals were untreated or given Hanks' solution only. Development of leukemia was assessed by spleen weight assay, as described elsewhere (6, 8).

Macrophage migration inhibition assay. Cellular immunity to the mycobacteria was determined after injecting the mice subcutaneously with 0.2-ml quantities of Freund's complete adjuvant in 3–4 sites approximately 3 weeks before testing. At the time of assay the animals were killed by cervical disloca-

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[2] Present address: Department of Microbiology, College of Science, Pennsylvania State University, University Park, Penn.

TABLE I. Areas of Cell Migration (mm^2) from Capillary Tube Cultures Containing Peritoneal Cells from Normal and Friend Leukemia Virus-Infected Mice After Immunization with Freund's Complete Adjuvant.

	Unimmunized			Immunized[a]		
Mouse group	No PPD	+ PPD	% Migration[c]	No PPD	+ PPD	% Migration[c]
Normal	13.5	12.9	95.6	16.5	11.3	68.5
	15.5	16.2	104.5	17.5	10.1	57.7
	13.9	12.5	89.9	18.3	9.8	53.5
	14.5	14.9	102.8	16.2	7.1	43.8
	18.6	16.5	88.7	17.5	8.5	48.6
	19.3	17.5	92.8	18.5	10.2	55.1
	15.5	14.2	91.6	18.3	10.6	57.9
	16.9	17.5	103.6	16.3	9.2	56.4
	18.1	17.3	95.6	16.5	9.8	59.4
FLV infected[b]	12.8	13.3	103.9	11.3	10.9	96.5
	15.5	14.2	91.6	14.5	15.1	104.1
	17.5	17.1	97.7	13.8	14.6	105.8
	13.8	12.1	87.7	15.2	14.1	92.8
	15.2	16.5	108.6	16.5	14.9	90.3
	16.5	14.9	90.3	14.2	13.8	97.2
	12.2	11.5	94.3	13.2	14.9	112.9
	15.2	16.3	107.2	14.1	15.0	106.4

[a] Mice injected 14–15 days previously with Freund's adjuvant containing mycobacteria.
[b] Mice infected with virus 3 weeks earlier (1 week before immunization).
[c] Percentage of migration area of cultures without PPD; average of 3–4 cultures/mouse.

tion and the cells present in the peritoneal cavity were harvested by washing several times with cold sterile saline through an abdominal wall incision. Approximately 5–10 $\times$ 10^6 nucleated cells were obtained in 5 to 10 ml of saline, of which 70 to 80% were morphologically similar to macrophages. These cells rapidly phagocytized carbon particles *in vitro*. Similar numbers of cells were obtained from both control and infected mice. Each cell suspension was washed 2 to 3 times with saline by centrifugation in the cold and then suspended in a small amount of medium 199 containing 20% fetal calf serum. The cells were then placed in several capillary tubes, approximately 0.7 mm in diameter and 75–100 mm long (15). The tubes were sealed with melted paraffin and centrifuged at 500*g* in the cold. The tubes were then cut at the cell–medium interface and the bottom portion was placed into Sykes-Moore culture chambers, as described elsewhere (15). Control chambers contained medium alone; whereas the test chambers also contained 10–20 μg of purified protein derivative (PPD). The chambers were incubated for 18 to 24 hr at 37° and then examined for cell migration. The area of migration was determined by planimetry and the results were expressed as the percentage of migration area of the experimental tubes with antigen in comparison to the migration area in the uninhibited control chambers.

Results. Normal migration of peritoneal cells from nonleukemic mice immunized with Freund's complete adjuvant was readily inhibited *in vitro* (Table I). Addition of PPD to cell suspensions from immunized mice resulted in a significant decrease in the average area of migration, generally in the range of 40 to 60%. Addition of PPD to chambers containing peritoneal cells from normal, nonimmunized mice resulted in no inhibition of migration, as compared to migration of cells cultured without antigen. Migration inhibition became evident only when cells were obtained at least 8–12 days after sensitization. The inhibition reaction appeared max-

TABLE II. Effect of Time of Infection, Relative to Day of Sensitization with Mycobacteria, on Cellular Immunity of Peritoneal Cells to PPD in Capillary Tube Cultures.

Mouse group[a]	Migration area (mm^2)[b]		
	No PPD	+ PPD	% Migration
Normal	17.8 ± 2.5	10.1 ± 2.1	56.7
Virus infected, same day	14.8 ± 3.2	11.1 ± 2.6	75.0
3 days	12.5 ± 3.0	10.3 ± 5.3	82.4
8 days	16.7 ± 2.9	15.2 ± 3.8	91.0
15 days	14.8 ± 3.6	14.1 ± 4.2	95.3

[a] Groups of 4–8 mice, either normal or infected on day indicated, injected with complete Freund's adjuvant; all mice tested 14–15 days later by migration assay.

[b] Average of 4–6 duplicate cultures.

imal when mice had been sensitized for 2–3 weeks before sacrifice. This "base line" of migration inhibition was then used to determine the effects of FLV-induced leukemia on cellular immunity to the mycobacteria. Mice infected 1 week earlier with FLV were injected with Freund's complete adjuvant. Two weeks later (the 3rd week of the disease) peritoneal cells were obtained and tested for migration activity in the presence or absence of PPD. PPD did not affect the area of migration of these cells (Table I). There was no significant difference between the average area of cell migration from chambers containing peritoneal cells from either untreated or antigen-injected FLV-infected mice, regardless of the presence of PPD.

Since development of overt leukemic symptoms occurred rapidly, even with a relatively low virus dose (7, 9), it was not feasible to assess the effect of FLV infection on early events during development of cellular immunity. Nevertheless, it seemed of value to study the effect of virus infection, relative to day of sensitization with the mycobacterial antigen, by the migration-inhibition assay. Therefore, groups of mice were infected with FLV and then injected with the adjuvant either on the same day or 3, 8, or 15 days later. All mice were assayed 14–15 days after sensitization. Thus, the duration of infection for each mouse group varied, but the time between immunization and assay was constant. As shown in Table II, almost normal migration occurred with cells obtained from mice infected 15 days before antigen sensitization, regardless of the presence of PPD. Slightly less migration occurred when the cells were obtained from mice sensitized 3 or 8 days after infection and incubated with PPD. Less migration was noted with cells obtained from mice sensitized the same day as immunization, although this inhibition was still significantly less than that occurring with control cells from noninfected mice sensitized weeks earlier.

All mice used for the above experiments, regardless of day of sensitization, already exhibited a large spleen at the time of assay (at least 2 weeks after infection). Thus, an attempt was made to determine the effect of virus infection on cellular immunity induced *before* injection of the virus. For this purpose mice were injected with the adjuvant and then infected with FLV 3, 8, or 15 days before sacrifice. Therefore, all of the mice were sensitized 3 weeks before assay, but the day of infection varied. As shown in Table III, there was a significant level of migration inhibition only with cells obtained from the mice infected 3 days before sacrifice. There was only a suggestive degree of migration inhibition with cells from mice infected 8 and 15 days before sacrifice (13 and 6 days after immunization, respectively).

Discussion. The results of this study support the concept that leukemogenesis induced by a virus such as FLV results in a marked inhibition of cellular as well as humoral immunity. Injection of this virus into susceptible BALB/c mice results in rapid development of splenomegaly and blood cell dyscrasia, culminating in erythroblastic leukemia (3, 6). Splenomegaly becomes evident

TABLE III. Effect of Infection with FLV on Cellular Immunity to Mycobacteria for Mice Sensitized 3 Weeks Earlier with Freund's Complete Adjuvant.

Day of infection relative to day of assay[a]	Migration area (mm^2)[b]		
	No PPD	+ PPD	% Migration
None (saline controls)	18.5 ± 3.6	11.3 ± 2.5	61.1
+3 days	18.2 ± 4.9	12.1 ± 3.2	66.5
+8 days	16.2 ± 2.6	13.3 ± 4.3	82.1
+15 days	17.3 ± 4.9	15.2 ± 4.0	87.9

[a] Groups of 5–6 mice either untreated or infected with FLV on day indicated relative to day of sacrifice; all mice sensitized with adjuvant 3 weeks before assay.

[b] Average migration areas of 4–6 cultures from pooled peritoneal cells from each mouse group.

generally after the first week, and the animals develop many overt symptoms during the second week. Cellular immunity, as assessed by the macrophage migration-inhibition procedure, appeared to be markedly suppressed during the earliest days of the disease. The ability of peritioneal exudate cells from these mice to react with PPD *in vitro* was strikingly affected. Similar suppressive effects on antibody forming cells have been observed (4, 6–10). Thus, FLV, as well as other murine leukemogenic viruses, may be useful as a "molecular probe" to inhibit cellular as well as humoral immune responses (2–8).

Normal control mice, when sensitized with Freund's adjuvant containing mycobacteria, readily developed cellular immunity as assessed by the migration inhibition assay. This procedure is considered to be an *in vitro* correlate of delayed hypersensitivity, *i.e.*, cellular immunity. Impaired cellular immunity of leukemic mice could be demonstrated, however, only late in the disease. In the first series of experiments mice were sensitized shortly after infection. However, development of optimum sensitization, as assessed by the *in vitro* assay (as well as by other criteria) required at least 2 weeks. Thus, at the time of assay, all of the mice exhibited gross signs of leukemia and disease. The effect of FLV on the early stages of development of cellular immunity could not be assayed directly in this model. In an alternate approach, the early effects of FLV infection was studied in mice sensitized *before* virus administration. The mice in these experiments had either developed hypersensitivity to the mycobacteria or were undergoing sensitization. Infection of these animals with FLV still resulted in suppression of the migration-inhibition reaction.

The cells involved in both the defect in cellular immunity as well as in leukemogenesis are not known. It is widely believed that migration inhibition occurs because of release of a migration inhibition factor (MIF), or other factors, from "sensitized" lymphocytes interacting with specific antigen (13, 14). The macrophages in this system are considered to be only the "indifferent" indicator of the reaction (13, 14). Thus, the results of the present study can be interpreted as demonstrating the absence of "sensitized" lymphocytes in the peritoneal cells of leukemic mice. Glass adsorption and other experiments are in progress with peritoneal cells to determine if macrophages from infected mice can respond to MIF produced by lymphocytes from normal sensitized animals exposed to PPD. Other experiments are underway to compare MIF release from spleen and lymph node cells from both normal control and FLV-infected mice sensitized to mycobacteria. However, in early experiments no evidence of MIF production by spleen cells has been obtained. Thus it appears that spleen cells, presumably the major target of the virus, may lose their capacity to interact with antigen to produce MIF, as assessed by the indirect macrophage migration procedure. In this regard, Bendinelli (17) recently reported that peritoneal cells from FLV-infected mice have lost much of their ability to form antibody *in vitro* when exposed to sheep erythrocytes, as occurs with peritoneal cells from normal mice similarly incubated with the erythrocytes. The comparatively similar results described here with the delayed hypersensitivity test *in vitro* suggests a close correlation between a defect in cellular immunity and humoral antibody formation due to infection with a leukemogenic virus.

Summary. Cellular immunity in leukemic mice was studied using Friend leukemia virus and susceptible BALB/c mice. Injection of this virus into the test mice resulted in rapid development of splenomegaly and other symptoms of leukemia. There was a concomitant decrease in the ability of peritoneal cells from these animals to respond to PPD *in vitro*, as assessed by the macrophage migration-inhibition procedure.

Sensitization of normal mice with mycobacteria in Freund's complete adjuvant resulted in rapid development of cellular immunity as assessed by the migration-inhibition assay. Failure to detect cellular immunity by this assay with peritoneal cells from leukemia virus-infected mice correlates with the marked decrease in humoral immunity, as well as cellular immunity previously assessed by skin graft rejection assays.

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