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Immunosuppression *in Vitro* Induced by Leukemia Virus-infected Splenocytes[1] (38544)

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Earlier studies reported from this laboratory and by other investigators have demonstrated that infection of mice with Friend leukemia virus (FLV) results in a marked immunosuppression to a variety of antigens, including erythrocytes, bacterial, and transplantation antigens (1–3). For mice injected with sheep erythrocytes (SRBC) fewer antibody-forming cells develop in virus-infected mice as compared to normal controls immunized with the same dose of antigen. Immunosuppression is also evident *in vitro*, since cultures of spleen cells from FLV-infected mice do not respond to SRBC as well as spleen cells from normal mice (4).

In order to study the effects of FLV infection in a completely *in vitro* system, attempts were made in the past to inhibit not only the maintenance but also the induction of antibody formation by FLV, using tissue culture models. However, addition of cell-free FLV preparations to normal spleen cell suspensions *in vitro*, as well as spleen cells from SRBC-primed donor mice, failed to affect the appearance of antibody plaque-forming cells (PFC) (4). The same virus preparation, however, readily induced symptoms of leukemia and suppressed antibody formation to SRBC when injected into mice. Thus it appeared that virus *per se* did not influence antibody formation in the *in vitro* model system.

In the present study it was found that viable spleen cells from FLV-infected mice, when added to cultures of spleen cells from normal syngeneic mice, markedly suppressed the expected induction of antibody to SRBC during *in vitro* immunization. Although viable leukemia cells were necessary for such an effect, it seems likely that FLV or an FLV-associated product being released from the infected cells was responsible for the immunosuppression.

Materials and methods. Experimental animals. Inbred Balb/c mice were obtained from Cumberland View Farms, Clinton, TN. They were approximately 20 g in weight at the initiation of an experiment and were housed in groups of 8–10 in plastic mouse cages; they were fed Purina mouse pellets and water *ad libitum.*

Leukemia virus. A stock preparation of FLV was used for these experiments exactly as described previously (5). The virus was free of lactic dehydrogenase virus. Immediately before use, an aliquot of the virus preparation was thawed and appropriate dilutions made in sterile Hanks' balanced salt solution (BSS). A 10^{-1} dilution contained approximately 5000 ID_{50} virus per ml.

Virus infection. Varying concentrations of the stock FLV preparation was given in 0.2 ml volumes intravenously (iv) to infect Balb/c mice. For *in vitro* infection, 0.1 ml of the virus preparation was added to 0.5 ml suspensions of spleen cells in tissue culture.

Anti-FLV serum. Normal Balb/c mice were immunized with formalinized infected spleen homogenates in complete Freund's adjuvant to stimulate formation of anti-FLV neutralizing antibody (6). A 1:50 dilution of this serum mixed with a highly infectious dose of FLV completely protected Balb/c mice.

Tissue culture preparations. Marbrook tissue culture vessels (Bioresearch Glass Co., Vineland, NJ) were used in these studies. Individual vessels contained five million nucleated splenocytes from normal Balb/c mice. Cells were cultured on a dialysis membrane in 12 ml minimal essential medium (MEM) supplemented with 10% fetal calf serum (pH 7.2). In some experiments double Marbrook vessels were used. Nucleopore membranes (0.45 μm) were used to separate

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leukemic spleen cells from normal spleen cells in the double vessels. After preparation, all cell cultures were incubated in an atmosphere of 83% N_2, 10% CO_2 and 7% O_2 in a 37° incubator.

In vitro immunization. SRBC obtained in Alsever's solution were washed several times with cold MEM and a standard concentration of 1×10^8 erythrocytes/ml prepared; 0.02 ml of the stock preparation (2×10^6 SRBC) was added to 5×10^6 normal spleen cells for immunization *in vitro*.

Hemolytic plaque assay. Spleen cells in individual Marbrook chambers were harvested on the fifth day after culture initiation and assayed for antibody PFC by the standard hemolytic plaque technique in agar gel (7). In brief, aliquots of the cells were mixed with 1.0 ml melted agarose (0.35%) and 0.1 ml of a freshly washed suspension of 10% SRBC. The mixture was poured onto a 15×60 mm diameter Petri dish containing a previously prepared base layer of agarose. After a 1-hr incubation at 37°, the test plates were treated with 1.0 ml sterile guinea pig serum diluted 1:10 with Hanks' BSS as the source of complement. The plates were reincubated for an additional hr at 37° and the number of hemolytic PFC appearing on each plate was enumerated.

Leukemic splenocytes. For inhibition experiments infected splenocytes from Balb/c mice 7–10 days after FLV infection (0.2 ml of 10^{-2} FLV given iv) were added to individual culture chambers at the time of culture initiation as described in Results.

Results. Addition of cell-free homogenates of FLV to spleen cell suspensions from normal mice at the time of *in vitro* immunization with SRBC failed to influence the expected PFC response. As can be seen in Table I, large numbers of PFC appeared in control cultures containing splenocytes from normal mice immunized *in vitro* with SRBC (group A). When varying dilutions of the infected spleen homogenate were added to cultures of normal spleen cells, there was no significant effect on the PFC response (groups B–E). However, the virus used was capable of infecting normal mice, as evidenced by the rapid development of splenomegaly after an injection of a 10^{-1}–10^{-4} dilution (5). Spleen cells from mice infected 7–10 days earlier with a 10^{-2} dilution of FLV markedly inhibited the expected PFC response of normal splenocytes immunized with SRBC *in vitro* (groups F–I). Spleen cell cultures incubated with 10^5 or 10^6 infected cells contained only 20–30% the number of PFC of control, uninfected cultures. A concentration of 10^4 infected spleen cells resulted in approximately a 50% inhibition of the PFC response. No significant inhibition occurred, however, when 10^3 infected spleen cells were added. Thus a ratio of one infected spleen cell to either 5 or 50 times more normal spleen cells (groups F and G) significantly depressed PFC responsiveness, whereas one infected cell for every 500 or more normal splenocytes (groups H and I), resulted in moderate to no inhibition.

Suppression of PFC formation occurred only with viable cells from infected animals; heating the leukemia cell suspension for 30 min at 56° abolished the immunosuppressive activity of the leukemic splenocytes (Table

TABLE I. EFFECT OF SPLEEN CELLS FROM FLV-INFECTED MICE OR FLV HOMOGENATES ON *IN VITRO* IMMUNIZATION OF NORMAL MOUSE SPLEEN CELLS TO SHEEP ERYTHROCYTES.

Group	FLV preparation added to cultures[a]		PFC/culture[b]	Percent of control
A	None (controls)		3500 ± 477	—
B	FLV homogenate	10^{-1}	3680 ± 445	105.1
C		10^{-2}	3020 ± 225	86.3
D		10^{-3}	3240 ± 406	92.6
E		10^{-4}	3300 ± 391	94.3
F	Infected spleen cells	10^{6}	860 ± 208	24.6
G		10^{5}	930 ± 375	26.7
H		10^{4}	1840 ± 321	52.6
I		10^{3}	3750 ± 528	107.1

[a] Indicated concentration of spleen cell or splenic homogenate from Balb/c mice infected 10 days earlier added to 5×10^6 spleen cells from normal mice immunized *in vitro* with 2×10^6 SRBC.

[b] Average number of hemolytic PFC for four to six cultures 5 days after *in vitro* immunization and culture initiation.

TABLE II. EFFECTS OF VARIOUS TREATMENTS OF FLV-INFECTED SPLEEN CELLS ON IMMUNOSUPPRESSION OF SPLEEN CELLS FROM NORMAL MICE IMMUNIZED *IN VITRO* WITH SHEEP ERYTHROCYTES.

Treatment of FLV-spleen cells[a]	PFC/culture[b]	Percent of control
None (untreated)	1250 ± 281	36.1
Heated (56°, 30 min)	3580 ± 548	103.1
Anti-FLV serum (1:10)	3340 ± 463	96.3
Normal serum	1340 ± 305	38.6
Separated by filter (0.45 μm)	1450 ± 247	41.8
Separated by filter plus anti-FLV serum	3860 ± 621	111.2

[a] Spleen cells from mice injected iv 7–10 days earlier with 10^{-2} concentration of FLV treated as indicated (see text) before incubation with normal spleen cells *in vitro*.

[b] Average number of PFC for four to six cultures 5 days after *in vitro* immunization of 5×10^6 spleen cells with 2×10^6 SRBC and incubation with 10^5 spleen cells from FLV-infected mice treated as indicated; PFC for control cultures without infected splenocytes = 3470 ± 435.

II). This indicated that living cells were necessary for the *in vitro* immunosuppression. However, the leukemic splenocytes did not have to be in direct contact with the normal spleen cells to suppress their responsiveness to SRBC. Separation of the two spleen cell populations by a nucleopore filter (0.45 μm diameter pore size), using a double Marbrook culture vessel, still resulted in significant immunosuppression (58%) (Table II). Thus, it seemed likely that a "factor" from infected spleen cells inhibited the antibody response. Presumably this subcellular material was virus, since specific anti-FLV serum, either when added directly to the infected splenocytes in one chamber of the culture vessel or when added to the other chamber containing the normal splenocytes, neutralized the inhibitory effects of the leukemic splenocytes (Table II). Normal serum or serum with specificity to other antigens did not influence the immunosuppressive properties of the leukemic splenocytes.

Discussion. Antibody-forming cells can be readily induced *in vitro* when spleen cells from normal mice are exposed to SRBC. The present experiments indicate that leukemic spleen cells derived from mice infected with FLV can markedly inhibit such *in vitro* immunization. Earlier studies from this and other laboratories showed that FLV, when injected directly into mice prior to challenge immunization with SRBC, suppressed the antibody response to SRBC and other antigens (1–5, 8–10). However, the mechanisms whereby FLV or other tumor virus impairs immune competence is still unknown.

As found in earlier preliminary studies and confirmed in the present study, addition of cell-free FLV preparations which have marked immunosuppressive properties *in vivo* did not inhibit the immune response of normal spleen cells immunized *in vitro*. Leukemic spleen cells from FLV-infected mice were, however, fully capable of suppressing antibody formation *in vitro*. Viability of leukemic cells was a necessity, since heat-killed spleen cells had no effect. Immunosuppression also resulted when leukemic cells were separated from SRBC-immunized spleen cells by a cell-impermeable nucleopore filter (0.45 μm). On the other hand, dialysis membranes, when used to separate infected from normal cells, prevented the immunosuppression (unpublished observation). Dialysis membranes are impervious to viruses the size of FLV, but nucleopore membranes are not. These results suggest that the suppressive properties of the splenocytes could have been due to FLV *per se* or an FLV-induced or associated product of the infected cells and not to the direct action of the infected lymphoid cells on the target splenocytes. Experiments using anti-FLV serum support this concept. Anti-FLV serum, when added to the leukemic cells in a double Marbrook vessel or the "target" normal splenocytes, effectively abolished the immunosuppression observed in cultures without anti-FLV serum or those containing normal serum.

An obvious question arises as to why infected splenocytes or a product derived from these cells induced immunosuppression, but virus homogenates (i.e. stock virus preparations) did not. Even large concentrations of cell-free virus (10^0 dilution) had no suppressive effect, whereas as few as 10^4 infected

cells markedly inhibited the *in vitro* response of normal spleen cells to SRBC. One can presume that virus obtained from spleen homogenates would be present in much higher titer than virus released directly from as few as 10^4 cells *in vitro*. However, it is also likely that the virus homogenates contain not only infectious virus, but also much noninfectious virus particles and possibly virus associated antigens. Noninfectious virus might compete for possible virus-specific receptors on the normal immunocytes and thus prevent virus-induced immunosuppression. In contrast, virus or virus-associated factors presumably released directly from infected splenocytes in culture may be more effective inhibitors of normal splenocytes.

It is also possible that "accessory" factors may be needed for FLV infection of leukocytes and these may be absent or non-functional *in vitro*. Such postulated factors may be supplied by other host cells during *in vivo* infection. Further studies concerning a possible role for such postulated accessory factors seem warranted. However, regardless of the mechanisms involved, it seems apparent from the present study that immunosuppression can be induced *in vitro* when spleen cells from FLV-infected mice are incubated with normal splenocytes. This model system should permit further analyses of various parameters involved in the immunodepression associated with murine leukemia virus infections.

Summary. Immunization of dispersed spleen cells from normal mice *in vitro* with SRBC was suppressed by simultaneous incubation of the spleen cell cultures with splenocytes from mice previously infected with FLV. Cell-free virus preparations alone did not suppress the antibody response. In contrast, relatively small numbers of splenocytes from infected mice, even when present at a ratio of 1–500 normal spleen cells, significantly suppressed the *in vitro* immune response to SRBC. Viable leukemic splenocytes were necessary for immunosuppression although the leukemic cells did not have to be in direct contact with the normal spleen cells. Specific anti-FLV serum, when added to the leukemic splenocytes or to normal spleen cells separated from infected cells by cell-impermeable membranes, prevented immunodepression.

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