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Restoration of Leukemia Virus-Suppressed Immunocytes *In Vitro* by Peritoneal Exudate Cells[1] (39166)

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Recent studies concerning the mechanism of immunosuppression induced by oncornaviruses such as Friend leukemia virus (FLV) have focused attention on the various cell classes involved in impaired immunologic function. For example, cell transfer studies have suggested that antibody precursor cells, rather than antibody-forming cells per se, may be the major target for immunosuppressive leukemia viruses (1–3). Transfer of bone marrow cells from FLV-infected mice, together with thymocytes from normal mice, resulted in markedly depressed immune responses in irradiated recipients. In contrast, thymus cells from infected mice, at least early during the course of FLV-induced leukemia, collaborated normally with bone marrow cells from noninfected animals, indicating that the "helper" cell function of the thymocytes was not impaired (3, 4).

It is also plausible that macrophages involved in the initial stages of antibody formation to an antigen such as sheep erythrocytes, presumably by "processing" or "focusing" the antigen, might be affected adversely by FLV, resulting in immunosuppression. However, a recent study in this laboratory showed that peritoneal exudate cell preparations rich in macrophages failed to restore normal immunoresponsiveness when injected into FLV-infected mice (5). It is apparent, however, that *in vivo* conditions present a number of obvious obstacles to the analysis not only of cell types involved in normal immune responsiveness but also analysis of the complex interactions between an immunosuppressive virus such as FLV and individual lymphoid cells. Thus, the availability of completely *in vitro* methods for studying antibody formation provided a valuable alternative to the *in vivo* experiments with FLV. For example, other studies in this laboratory showed that splenocytes from FLV-infected mice remain unresponsive during *in vitro* immunization with sheep erythrocytes (6, 7). Whereas normal splenocytes were readily immunized *in vitro* to the erythrocyte antigen, spleen cells from mice infected with FLV prior to *in vitro* culture were markedly depressed in their immune responsiveness. In the present study, attempts to restore antibody-forming capability with different cell types revealed that unlike the *in vivo* situation, addition of peritoneal exudate cells from normal donor mice to FLV-suppressed splenocyte cultures resulted in restored antibody responsiveness.

Methods and materials. Experimental animals. Young adult male Balb/c mice, 6 to 8 weeks of age, were obtained from Cumberland View Farms, Clinton, Tennessee, and housed in groups of six to eight in plastic mouse cages. They were fed Purina mouse pellets and water *ad libitum.*

Leukemia virus. A stock preparation of FLV passaged in this laboratory for the last nine years was used for infection of Balb/c mice. The stock virus preparation consisted of clarified 10% spleen cell homogenates from 8–10 day infected Balb/c mice and contained approximately 10^4 ID_{50} virus doses per ml. The virus preparation was free of detectable lactic dehydrogenase and choriomeningitis viruses and consisted of both the lymphatic leukemia and spleen focus-forming virus components of the Friend complex.

Antigen. Sheep erythrocytes (SRBC) in Alsever's solution were obtained from Baltimore Biological Laboratories, Baltimore, Maryland. The erythrocytes were washed several times in saline and resuspended to a

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10% concentration (v/v). For *in vivo* immunization, the mice were injected with 0.2 ml of a 2% suspension of the RBC's. For *in vitro* immunization (see below), 0.1 ml of a 0.1% suspension of washed RBC was added to each culture.

In vitro immunization. The Marbrook culture system for immunization of dispersed cell suspensions was used for the *in vitro* studies (6, 7). The chambers were obtained from BioResearch Glassware Company, Vineland, New Jersey. Spleen cell suspensions were prepared either from normal or FLV-infected mice, as described previously (6, 7), and 5×10^6 washed splenocytes in 1.0 ml Minimal Essential Medium (MEM) fortified with 10% fetal calf serum (Flow Laboratories, Rockville, Md.) were placed on the dialysis membrane in the inner chamber, and 11 ml of medium were placed in the outer reservoir chamber. The vessels were incubated at 37° in an atmosphere of 5% CO_2 and 95% air.

Assessment of antibody-forming cells. Aliquots (0.1 ml) of dispersed spleen cell suspensions from mice or Marbrook culture chambers were added to 2.0 ml 1% melted agar gel (Difco Laboratories, Detroit, Michigan) containing 0.1 ml of a 10% suspension of sheep erythrocytes. The agar–cell mixture was quickly poured onto the surface of a previously prepared petri plate containing a base layer of 10 ml solidified agar. The plates were incubated at 37° for 1 hr and then treated with 2 to 3 ml of a 1:10 dilution of guinea pig complement. After further incubation at 37° for 1 hr, the numbers of antibody plaque-forming cells (PFC) were enumerated exactly as described earlier (6–8). The numbers of plaques on three or more plates containing splenocytes from a single cell suspension were counted and used to determine the number of plaque-forming cells (PFC) per million splenocytes plated or per whole spleen or Marbrook culture.

Peritoneal exudate cells. Normal mice were killed 3–5 days after injection with 1.0 ml 2% proteose-peptone (Difco Laboratories, Detroit, Michigan). The peritoneal exudate cells were obtained by washing the peritoneum with 5 to 10 ml MEM. The resulting cell suspensions were washed by low speed centrifugation in the cold several times, and the cells were resuspended to a concentration of 5×10^7 nucleated cells per ml. Approximately 75 to 80% of the cells were morphologically similar to macrophages and readily phagocytized sheep erythrocytes or carbon particles.

Experimental results. Immunization of FLV-infected mice resulted in development of markedly fewer antibody-forming cells as compared to the responses of noninfected mice. The degree of immunosuppression was directly related to the time interval between infection of the donor mice and challenge immunization. Splenocyte cultures prepared from infected mice failed to respond normally to sheep erythrocytes when immunized *in vitro* in Marbrook chambers, as compared to the responses of splenocytes from normal donor animals immunized *in vitro* in the same way (Table I). For example, on the seventh day after infection of mice with a 10^{-1} dose of FLV, there were 90 to 95% fewer PFC's in the spleens of FLV-infected vs control mice. Similarly, spleen cells from these infected mice produced very few PFC's to SRBC *in vitro* as compared to the much larger number of PFC's in similar cultures from normal mice (Table I). The time of peak response for PFC production by splenocytes from normal mice immunized either *in vivo* or *in vitro* was on Day 5. Although the number of PFC produced by spleen cells from infected mice was much reduced, the peak response was also on Day 5.

Addition of PE cells to Marbrook chambers containing the immunosuppressed splenocytes from infected mice resulted in varying numbers of PFC's. Addition of 10^3 or 10^4 PE cells did not alter the PFC response. In contrast, addition of 10^5 to 10^6 PE cells to spleen cell cultures from infected mice resulted in markedly higher PFC responses. Optimum responses occurred with the 3×10^5 PE cell dose. This number of PE cells resulted in a PFC response essentially similar to that observed in chambers containing normal spleen cells. Addition of PE cells to the control spleen cell cultures had very little effect on the expected PFC response except when 10^6 or more cells were used. The larger numbers of PE cells con-

TABLE I. EFFECT OF INFECTION OF DONOR MICE WITH FLV ON ANTIBODY FORMATION BY SPLEEN CELLS *IN VIVO* OR AFTER *IN VITRO* IMMUNIZATION WITH SRBC.

Time (days) after infection of mice with FLV[a]	*In vivo* response[b]		*In vitro* response[c]	
	Per 10^6 splenocytes	Percent of control	Per 10^6 splenocytes	Percent of control
None (controls)	478 ± 55	—	892 ± 75	—
−1	410 ± 47	85.8	635 ± 48	71.5
−3	112 ± 29	23.4	195 ± 11	21.9
−7	18 ± 5	3.8	25 ± 6	2.9
−10	7 ± 3	1.5	12 ± 2	1.3
−15	5	1.0	9 ± 2	1.0

[a] Group of mice injected iv with 10^{-1} dose of FLV on indicated day before testing for PFC responsiveness to SRBC.

[b] Average number of PFCs (± SD) for spleen cells from five to six mice per group 5 days after *in vivo* challenge immunization with SRBC.

[c] Average number of PFCs (± SD) for four to six Marbrook cultures containing 5×10^6 spleen cells from indicated mouse group 5 days after *in vitro* immunization with SRBC.

sistently reduced the PFC response of normal cell cultures. No enhancement was observed with any PE cell concentration in control cultures. Optimum restoration of PFC formation occurred when the cells were added to spleen cell cultures from FLV-infected mice at the time of culture initiation. Addition of PE cells to the cultures 1 or 2 days after *in vitro* immunization had little if any influence on PFC formation, suggesting that the restorative effect occurred only early during the course of immunization. Addition of splenocytes, rather than PE cells, to the Marbrook chambers containing infected spleen cells had very little effect on the PFC response. This ineffectiveness of normal spleen cells was consistent with an earlier study where it was found that even small numbers of infected splenocytes could prevent the normal immune response of many more uninfected spleen cells (6). Furthermore, addition of glass adherent splenocytes, which contained many more macrophages than the unfractionated spleen cell preparations, also had no restorative effect.

TABLE II. EFFECT OF PE CELLS FROM NORMAL MICE ON PFC RESPONSIVENESS OF SPLEEN CELL CULTURES FROM NORMAL AND FLV-INFECTED MICE IMMUNIZED *IN VITRO* WITH SRBC.

Number of PE cells added per culture[a]	PFC response per 10^6 spleen cells[b]	
	FLV-infected[c]	Noninfected
None	62 ± 27	612 ± 80
3×10^3	123 ± 59	645 ± 138
3×10^4	168 ± 54	690 ± 180
10^5	361 ± 136	640 ± 150
3×10^5	386 ± 204	593 ± 192
10^6	279 ± 111	442 ± 163

[a] Cultures of 5×10^6 spleen cells from either FLV-infected or normal mice incubated in Marbrook chambers with indicated number of PE cells and immunized with SRBC.

[b] Average response of four to eight cultures per group 5 days after *in vitro* immunization of spleen cells from infected or normal mice.

[c] Donor mice infected iv with 10^{-1} dose of FLV 7 days before obtaining spleen cells for culture.

Discussion. The results of this study indicate that the *in vitro* immunosuppression of splenocytes from FLV-infected mice can be readily reversed by addition of PE cell suspensions from normal donor mice. This finding contrasts with results of a previous study indicating that injection of similar PE cell preparations directly into FLV-infected mice failed to restore immunocompetence to the same RBC antigens (5). In the *in vitro* situation, splenocytes from infected animals, although markedly impaired in their ability to respond to sheep RBC's as compared to the response of normal spleen cells, regained their ability to do so in the presence of PE cells. The PE cell suspensions may have provided a population rich in "activated" macrophages, which either directly or indirectly influenced the antibody responsiveness of the immunosuppressed spleen cells from infected animals.

This seems likely since more than 80% of the morphologically identifiable PE cells appeared to be macrophages. Furthermore, results of other studies (unpublished) indicated the PE cells harvested from unstimulated mice had little, if any, restorative effect. Fewer than 40% of the cells in such cell suspensions appeared to be macrophages. Spleen cell suspensions from normal mice, which also contain macrophages, were also ineffective, even when added in larger numbers. In addition, glass adherent cells prepared from normal spleen cell suspensions were without effect. Such ineffectiveness of splenic macrophages might be due to the absence of activation of these cells in the spleen; injection of proteose-peptone intraperitoneally probably results in "activation" of macrophages only in the peritoneum and not the spleen or other lymphoid tissues.

A related study in this laboratory with a lymphatic leukemia virus (Rowson–Parr virus) resulted in essentially similar restoration of antibody formation with PE cells (9, 10). *In vitro* cultures of spleen cells from such leukemia-virus infected mice were immunodeficient, but showed essentially normal immune responsiveness upon addition of relatively small numbers of PE cells. The Rowson–Parr virus was obtained originally by end-point dilution procedures from the spleens of FLV-infected mice. This virus causes very little if any splenomegaly in susceptible mice during the first few weeks of infection. Addition of 10^5 PE cells from proteose-peptone-stimulated donor mice resulted in restoration of PFC responsiveness similar to that observed in the present study with FLV-suppressed splenocytes. Furthermore, treatment of the PE cells with anti-theta or anti-Ig serum plus complement before addition to the cultures to eliminate T- or B-lymphocytes failed to affect the immunologic restorative properties of the PE cells (9, 10).

The mechanism of restoration of PFC responsiveness by induced PE cells may be due to more optimum phagocytosis and/or processing of the RBC's in the *in vitro* cultures, so that the antigen may be presented to antibody precursor cells in a more efficient form. It is possible that the antigen-processing macrophages are suppressed in the spleen of FLV, as well as other leukemia virus-infected mice (3). In addition, an impairment of actual immunocytes is also likely. Nevertheless, addidtion of "normal" PE cells containing functional macrophages may provide the cultures with a more optimally "processed" antigen that can serve as a more efficient immunogen for the depressed numbers of antibody producing cells. A quantitative or qualitative immune defect in the cultures remaining, even after addition of PE cells, would account for the failure to induce full restoration of antibody responsiveness. This seems due to the absence of normal numbers of antigen reactive or antibody precursor cells in infected spleens. Even with optimum "processing" of RBC's by added PE cells, the absence of optimal numbers of such precursor cells would result in a less than maximum PFC response.

The results of the present study also indicate that a close contact between added PE cells and lymphocytes may occur *in vitro* as compared to *in vivo*. The earlier study showing that adoptive transfer of PE cells to FLV-infected mice failed to alter immunosuppression *in vivo* may be due to the possibility that the transferred PE cells did not reach the appropriate site and/or "home" normally into the recipient spleen. Thus, optimum contact between transferred PE cells and the depressed number of antibody precursor cells may not occur *in vivo*. The marked disruption of normal splenic architecture after FLV injection may also make interaction between transferred PE cells and resident lymphoid cells less than optimum. On the other hand, in the *in vitro* situation, even small numbers of antigen-reactive or antibody precursor cells might respond in a positive manner when normal PE cells are added to the cultures.

Although it seems likely that the primary immunological defect in FLV-infected animals is depletion of immunocytes and their precursors (3), the results of the present study suggest that impaired macrophage function (i.e., antigen processing activities) may also be important and that restoration of antibody responsiveness may occur when PE cell suspensions rich in macro-

phages from normal mice are added to cultures of spleen cells from infected mice. However, additional studies in progress with other cell types, including purified T- and B-cell preparation, should also be of value in elucidating the mechanism of immunodepression by leukemia viruses.

Summary. Depressed antibody responsiveness to sheep erythrocytes in mice infected with Friend leukemia virus continued *in vitro* when spleen cell cultures from infected animals were cultured in the presence of antigen. Addition of PE cells from normal donor mice to the immunologically depressed splenocyte cultures resulted in a marked restoration of antibody responsiveness. Restoration of the immune response was PE cell dose-dependent; a ratio of 1 PE cell per 10 splenocytes resulted in the largest numbers of PFC's. These results suggest that impaired antibody responsiveness by spleen cell cultures from FLV-infected mice may be due, in part, to effects on antigen-processing macrophages, since restoration of immune responsiveness occurs by PE cell supplements.

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