INFECTION AND IMMUNITY, Mar. 1983, p. 1260–1264
0019-9567/83/031260-05$02.00/0

Vol. 39, No. 3

# Role of Antibody Response Helper Factors in Immunosuppressive Effects of Friend Leukemia Virus

R. CHRISTOPHER BUTLER,[1]* JERI M. FRIER,[1] MRUNAL S. CHAPEKAR,[1] MARY O. GRAHAM,[1] AND HERMAN FRIEDMAN[2]

*Arlington Hospital, Arlington, Virginia 22205,[1] and University of South Florida, Tampa, Florida 33612[2]*

Received 2 July 1982/Accepted 5 November 1982

The primary antibody response of BALB/c splenocytes to sheep erythrocytes in vitro was suppressed by infection with Friend leukemia virus (FLV), with the response capacity decreasing with increasing duration of infection. The acquisition of normal antibody responses was amplified by macrophage-produced antibody response helper factor(s). FLV-infected mice were treated with bacterial lipopolysaccharide to induce the release of these helper factors into the serum. Similar to the loss of antibody response capacity by their splenocytes, the FLV-infected mice progressively lost the ability to produce helper factors in response to lipopolysaccharide. In vitro cultures of FLV-infected cells also showed a depressed ability to produce helper factor activity both spontaneously and in response to lipopolysaccharide stimulation. The reconstitution of normal levels of exogenous helper factors to FLV-infected splenocytes restored the antibody response to normal or even elevated levels. These studies indicate that the mechanism for suppression of antibody responses by FLV involves the depression of antibody response helper factor production.

Infection of mice with murine leukemia viruses often results in marked impairment of immune responses, including those mediated by B cells, T cells, or both (14). One such oncornavirus, Friend leukemia virus (FLV), has been demonstrated to markedly suppress the development of specific antibody responses by susceptible strains of mice (1, 3, 11, 13, 16, 17). In the past it was generally accepted that this impairment was due to the interaction of the virus with antibody-producing B cells or their precursors.

One problem with studying the mechanism of action of FLV is that it is really a complex of viruses, including an erythroblastic leukemia virus which causes erythroblast proliferation within the spleen and the Rowson-Parr lymphatic leukemia virus (16). The differential effects of these components may cause a marked alteration in the identifiable cell populations within the spleen, which complicates measurement of the effects of FLV on specific immunological function. One concern has been that the proliferation of erythroblastoid cells in the spleen causes a false immunosuppression due to dilutional effects. Numerous studies, however, have demonstrated that FLV does produce a true suppression of immune response mechanisms (1–4, 6, 8–11, 13, 16, 17).

The ability to initiate an antibody response must be related to the number and frequency of potential antibody-producing cells in the spleen. There is evidence that, despite the proliferation of nonlymphoid cells in FLV spleens, the frequency of potential antibody-producing cells is not depressed until a terminal condition is approached. For example, even though the active development of a specific antibody response to sheep erythrocyte antigens (SRBC) is suppressed, the background antibody response per $10^6$ cells of unsensitized FLV-infected splenocytes to both SRBC and *Escherichia coli* is elevated early in the disease and is not depressed below normal levels for at least 18 days after infection (11). Furthermore, although splenocyte cultures infected with FLV in vitro also have a suppressed active antibody response, they display an elevated background antibody response, indicating an increased frequency of antibody response precursors during leukemic transformation (1). This blastogenic response of FLV-infected antibody response precursor cells could account for the observed maintenance of background antibody levels in vivo.

Recent studies have shown that the antibody response capacity of leukemic splenocytes can be restored under appropriate conditions. For example, the in vitro treatment of FLV-infected splenocytes with immunostimulants such as lipopolysaccharides (LPS) or muramyl dipeptide can induce the development of a near-normal antibody response to SRBC (3). Another observation was that the addition of peritoneal exu-

TABLE 1. Suppression of the primary in vitro antibody response to SRBC by FLV

| Source of responder cells[a] | PFC/$10^6$ cells ± SE[b] | % Control (P) |
|---|---|---|
| Normal | 518 ± 36 | 100 |
| 7-day FLV | 463 ± 25 | 89 |
| 14-day FLV | 316 ± 22 | 61 (0.01) |
| 21-day FLV | 106 ± 15 | 20 (0.001) |

[a] Mice were pretreated with 100 $ID_{50}$ of FLV from 7 to 21 days before sacrifice.

[b] Cultures of normal or FLV-infected splenocytes were sensitized with SRBC in vitro. After 5 days, the cultures were collected and assayed for PFC.

date macrophages to splenocyte cultures derived from advanced leukemic mice could restore the antibody response capacity, thus demonstrating that the mechanism for this unresponsiveness might be related to these accessory cells (17).

Since the mechanism for the suppression of antibody response by FLV is now apparently not due solely to infection of B cells by the virus but may involve macrophages, we sought to determine the mechanism by which FLV might exert its suppressive effects via macrophages. In previous studies, we have demonstrated that the development of antibody responses by normal splenocytes is greatly enhanced by the production of antibody response helper factor(s) by macrophages (5). These factors are released "spontaneously" by cultured macrophages, but their production is greatly enhanced by treatment with LPS. This study was designed to determine the role of these antibody response helper factors in the mechanism of FLV suppression of the active development of an antibody response.

## MATERIALS AND METHODS

**Experimental animals.** Inbred male BALB/c mice, 6 to 8 weeks of age, were obtained from Cumberland View Farms, Clinton, Tenn. Mice were infected by intraperitoneal injection of a 100 50% infective dose ($ID_{50}$) of FLV contained in 0.1 ml of a 1% clarified homogenate of infected splenocytes. The virus had been maintained by passage through adult BALB/c mice and contained both the spleen focus-forming and lymphatic leukemia virus components of the Friend complex.

**LPS.** *Serratia marcescens* LPS (generously donated by A. Nowotny) was prepared by the trichloroacetic acid extraction procedure, as previously described (15).

**Antigen.** SRBC in Alsever's solution were obtained from BBL Microbiology Systems, Cockeysville, Md. The erythrocytes were washed several times in medium and resuspended to a 0.1% concentration.

**In vitro immunization.** Covered plastic Linbro plates were used as culture chambers. Spleen cells from normal or FLV-preinfected mice were washed in media, and the numbers of viable nucleated cells were determined by the trypan blue exclusion technique with a hemacytometer. A suspension of $8 \times 10^6$ viable splenocytes suspended in 2.0 ml of complete tissue culture medium enriched with a standard nutrient cocktail and 10% fetal bovine serum was cultured in the Linbro plate wells as described elsewhere (12). For in vitro immunization, 0.1 ml of the 0.1% suspension of SRBC was added to each culture (ca. $2 \times 10^6$ erythrocytes). All cultures were incubated for 5 days at 37°C in a humidified atmosphere containing 10% $CO_2$.

**Assay for antibody-forming cells.** The numbers of direct hemolytic plaque-forming cells (PFC) to SRBC were determined by the micromethod of Cunningham and Szenburg (7). The numbers of PFC were enumerated for at least 8 to 24 cultures prepared from two to four spleen cell preparations, and the average number of PFC per $10^6$ cells was calculated. In all cases, only direct non-facilitated plaques were enumerated, and these were considered to be due to 19S immunoglobulin M antibody-producing cells.

**Post-LPS serum.** Normal or FLV-infected mice were injected intraperitoneally with 20 μg of LPS and exsanguinated 2 h later by aseptic cardiac puncture. Serum was separated from the erythrocytes and kept on ice until used.

**In vitro factor production.** Suspensions of $10^7$ splenocytes per ml from normal or FLV-infected mice were incubated in RPMI 1640 plus 10% fetal bovine serum and antibiotics at 37°C under $CO_2$. Experimental cultures received 10 μg of LPS per ml at the time of culture initiation. Supernatants were collected after 5 days and either stored on ice or frozen at −70°C until tested.

**Antibody response helper factor assay.** The presence of antibody response helper factor activity was determined by adding 0.01 ml of post-LPS serum or 0.1 ml of stimulated culture supernatants to the in vitro antibody cultures at the time of sensitization with SRBC. The helper factor activity of each preparation was considered to be proportional to the degree of enhancement of the antibody response over that of the normal untreated control cultures.

## RESULTS

The basis for this study was the observation that splenocytes from FLV-infected mice have a decreased ability to mount a primary antibody response against SRBC. The results demonstrate that the ability to produce an antibody response decreased steadily with increasing duration of infection (Table 1).

Since we had previously demonstrated the essential role of macrophage-produced antibody response helper factor(s) in the induction of antibody responses (5), we questioned whether the decrease in response capacity might be linked to helper factor production. Antibody response helper factors are produced in vivo in response to LPS stimulation and are found at maximal concentrations in 2-h post-LPS serum. Table 2 shows that mice infected with FLV gradually lose the ability to produce these helper

TABLE 2. Effect of FLV infection on the production of antibody response helper factors in vivo in post-LPS serum

| Serum source[a] | Mouse treatment (2 h)[b] | PFC/$10^6$ cells ± SE[c] | % Control response (P) |
|---|---|---|---|
| None | | 1,240 ± 97 | 100 |
| Normal | | 1,144 ± 114 | 92 |
| Normal | LPS | 2,346 ± 230 | 189 (0.005) |
| 7-day FLV | | 1,025 ± 153 | 83 |
| 7-day FLV | LPS | 1,817 ± 265 | 147 (0.05) |
| 14-day FLV | | 1,100 ± 123 | 89 |
| 14-day FLV | LPS | 1,488 ± 290 | 120 |
| 21-day FLV | | 1,205 ± 201 | 97 |
| 21-day FLV | LPS | 1,315 ± 282 | 106 |

[a] Mice were preinfected with 100 $ID_{50}$ of FLV from 7 to 21 days before the collection of serum.

[b] Normal and FLV-infected mice received an intraperitoneal injection of 20 μg of LPS 2 h before the collection of serum.

[c] Cultures of 8 × $10^6$ normal BALB/c splenocytes were treated with 0.5% serum at the time of in vitro primary stimulation with SRBC. After 5 days, the cultures were collected and assayed for PFC.

factors. The effects of normal unstimulated serum did not change, indicating that the loss of activity of the post-LPS serum was not due to the presence of any concomitant immunosuppressive factors. The stimulatory effects of the post-LPS sera were not due to the presence of residual LPS, which has been demonstrated to be several orders of magnitude below a stimulatory concentration (5).

Table 3 further illustrates this loss of ability to produce helper factors. In this study, cultures of normal or FLV-infected splenocytes were incubated for 5 days alone or in the presence of LPS. As previously described (5), normal unstimulated splenocyte culture supernatants contain small amounts of helper factor activity. LPS stimulation of normal cells induced a much greater activity. As a control for the direct activity of the small amounts of residual LPS in the supernatants, a 2-h supernatant was also collected and tested. It produced 262 ± 32 PFC per $10^6$ cells, which was 128% of control (not significant). Other studies have similarly shown that the stimulatory effects of LPS-treated cultures are independent of residual LPS (2, 4, 5).

Similar to the results obtained in vivo, the in vitro cultures of FLV-infected cells showed a depressed ability to produce helper factor activity both spontaneously and in response to LPS stimulation. The increasing spleen weights of infected mice are an indication of the dramatic proliferative response resulting from FLV infection and transformation of cells and are an indication of the state of progression of the disease.

The supernatants from unstimulated FLV splenocyte cultures did not contain detectable immunosuppressive factors (Table 3). However, the possibility existed that the LPS stimulation could have enhanced the shedding of FLV into the culture supernatants. The in vitro infection of cells by the virus could potentially cause the observed lowered responsiveness. To preclude this possibility, supernatants from LPS-stimulated FLV splenocytes were filtered through a 0.05-μm (300,000 molecular weight) Nuclepore filter. This filtration did not alter the effects of the supernatants or the antibody response (Table 4). Previous studies have demonstrated that this filtration procedure renders an FLV suspension noninfectious and non-immunosuppressive (13).

Since FLV infection caused splenocyte cultures to lose the capacity to produce antibody response helper factor activity, it was important

TABLE 3. Effect of FLV infection on antibody helper factor production in vitro

| Source of cells for supernatant production[a] | Spleen wt (g) | Treatment of cells for supernatant production[b] | PFC/$10^6$ cells ± SE[c] | % Control (P) |
|---|---|---|---|---|
| None | | | 205 ± 30 | 100 |
| Normal | 0.155 | | 284 ± 36 | 139 (0.05) |
| Normal | 0.155 | LPS | 619 ± 68 | 302 (0.001) |
| 1-day FLV | 0.168 | | 302 ± 40 | 149 (0.05) |
| 1-day FLV | 0.168 | LPS | 584 ± 45 | 285 (0.001) |
| 7-day FLV | 0.245 | | 241 ± 15 | 118 |
| 7-day FLV | 0.245 | LPS | 385 ± 71 | 188 (0.05) |
| 21-day FLV | 2.281 | | 196 ± 16 | 98 |
| 21-day FLV | 2.281 | LPS | 243 ± 30 | 118 |

[a] Mice were preinfected with 100 $ID_{50}$ of FLV from 1 to 21 days before sacrifice.

[b] Cultures of $10^7$ splenocytes per ml were incubated for 5 days with or without 10 μg of LPS per ml.

[c] Cultures of 8 × $10^6$ normal BALB/c splenocytes were treated with 5% supernatant from the FLV splenocyte cultures at the time of in vitro primary stimulation with SRBC. After 5 days, the cultures were collected and assayed for PFC.

TABLE 4. Lowered stimulatory capacity of supernatants from FLV-infected splenocytes not due to transfer of FLV in the supernatant

| Source of cells for supernatant production[a] | 300,000 MW filtration[b] | PFC/$10^6$ cells ± SE[c] | % Control (P) |
|---|---|---|---|
| None | − | 132 ± 34 | 100 |
| Normal (LPS treated) | − | 398 ± 102 | 302 (0.001) |
| Normal (LPS treated) | + | 344 ± 30 | 261 (0.001) |
| 10-day FLV (LPS treated) | − | 232 ± 48 | 176 (0.005) |
| 10-day FLV (LPS treated) | + | 258 ± 86 | 195 (0.005) |
| 30-day FLV (LPS treated) | − | 186 ± 24 | 141 |
| 30-day FLV (LPS treated) | + | 164 ± 30 | 124 |

[a] Mice were preinfected with 100 $ID_{50}$ of FLV from 10 to 30 days before sacrifice. All cultures were treated with 10 μg of LPS per ml for 5 days.

[b] Supernatants were filtered through a 300,000 MW Nuclepore filter to remove infectious virus.

[c] Cultures of $8 \times 10^6$ normal BALB/c splenocytes were treated with 5% supernatant from the FLV splenocyte cultures at the time of in vitro primary stimulation with SRBC. After 5 days, the cultures were collected and assayed for PFC.

to determine whether the restoration of this helper factor activity to FLV splenocytes would affect the antibody response capacity. Table 5 demonstrates that the addition of exogenous helper factors to FLV splenocyte cultures restored the antibody response to normal or even slightly elevated levels.

## DISCUSSION

The immunosuppressive effects of FLV on antibody responses to SRBC have been demonstrated in vivo (11, 13) and in vitro (1–4, 16, 17). The results shown here illustrate the steady decrease in the ability of murine splenocytes to mount an antibody response to SRBC in vitro after infection with FLV. Although the mechanism for the in vivo suppression by FLV appears to be complex and could include such nonspecific factors as dilution of immunocytes with nonlymphoid cells, structural alterations within the spleen, and altered cell migration patterns, etc., in vitro studies have shown that FLV produces a true suppression of the immune response mechanism (1–5, 13, 16, 17). Previous studies have shown that impaired macrophage function plays a role in this immunosuppression (16, 17). This study has attempted to determine the mechanism by which this macrophage impairment is translated into a suppressive effect on the generation of the antibody response to SRBC.

TABLE 5. Reversal of FLV-induced immunosuppression by treatment with antibody response helper factors

| Supernatant source[a] | Responder cells[b] | PFC/$10^6$ cells ± SE[c] | % Control (P) |
|---|---|---|---|
| None | Normal | 696 ± 46 | 100 |
| None | FLV | 240 ± 68 | 34 (0.001) |
| Untreated | Normal | 678 ± 36 | 97 |
| Untreated | FLV | 324 ± 38 | 47 (0.001) |
| LPS treated | Normal | 1,379 ± 82 | 198 (0.01) |
| LPS treated | FLV | 962 ± 102 | 138 (0.05) |

[a] Cultures of $10^7$ normal BALB/c splenocytes per ml were incubated for 5 days with or without 10 μg of LPS per ml.

[b] Cultures of $8 \times 10^6$ normal or 21-day FLV-infected splenocytes (spleen weight = 2.537 g) were treated with 5% supernatant at the time of in vitro primary sensitization with SRBC.

[c] After 5 days, the cultures were collected and assayed for direct PFC.

Our previous studies have demonstrated the role of macrophage-produced helper factors in amplifying the antibody responses of normal splenocytes (5, 6, 8). The factor is produced by macrophages, and its target is a cell of bone marrow lineage. It can enhance SRBC antibody responses in the absence of mature T cells. A dose-response relationship exists between helper factor concentration and the magnitude of the antibody response (5).

These studies have established a strong relationship between FLV infection the loss of ability to produce antibody response helper factors either spontaneously or in response to stimulation with LPS. This decrease in helper factor activity was demonstrated to occur both in vivo (post-LPS serum) and in vitro (splenocyte culture supernatants). In view of our previous studies, it is feasible that the observed decrease in helper factor production by FLV-infected cells could be causally related to the depressed antibody response. If this were the case, then the addition of exogenous helper factor should restore the antibody response of FLV cells. The results in Table 5 show that this reversal does occur and that leukemic splenocytes retain the capacity to produce a normal antibody response under the condition of adequate levels of helper factor activity.

Previous studies have shown that the suppression of antibody responses in FLV-infected mice

is at least partially due to suboptimal macrophage activity (16, 17). They suggested that this might be due to a depression of the antigen-processing activity of macrophages. In contrast, our results indicate that it is the inability of FLV-infected mice or cultures to produce sufficient quantities of a specific macrophage product, an antibody response helper factor, which inhibits the development of antibody responses by leukemic splenocytes.

The depression of antibody response helper factor production in vitro could be due to a decrease in either the number or the activity level of macrophages in leukemic spleens. Other investigators have demonstrated the presence of morphologically normal macrophages in leukemic spleens (10) and have concluded that the depression of other macrophage functions, such as migration in culture, was due to suppression of macrophages rather than to dilution with nonmigrating cells (9). The suppression of helper factor production in vivo is strong evidence that FLV has an inhibitory effect on macrophage populations which would normally be capable of producing antibody helper factor in response to antigen or immunoadjuvant stimulation. It is conceivable that these macrophages are refractory due to the effects of direct infection, a depression of stimulatory factors from other cells, or prolonged stimulation by FLV viral antigens which might block receptors. These possibilities are currently being evaluated in our laboratories.

## ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant 7R23CA27415-02 from the National Cancer Institute, and by awards from the Elaine R. Shepard Foundation and K. S. Associates.

## LITERATURE CITED

1. **Bendinelli, M., and H. Friedman.** 1980. B and T lymphocyte activation by murine leukemia virus infection. Adv. Exp. Biol. Med. **121B**:91–97.
2. **Butler, R. C., and H. Friedman.** 1979. Leukemia virus-induced immunosuppression: reversal by subcellular factors. Ann. N.Y. Acad. Sci. **332**:446–450.
3. **Butler, R. C., and H. Friedman.** 1980. Restoration of leukemia cell immune responses by bacterial products, p. 1719–1720. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease. American Society for Microbiology, Washington, D.C.
4. **Butler, R. C., H. Friedman, and A. Nowotny.** 1980. Restoration of depressed antibody responses of leukemic splenocytes treated with LPS-induced factors. Adv. Exp. Biol. Med. **121A**:315–322.
5. **Butler, R. C., A. Nowotny, and H. Friedman.** 1979. Macrophage factors that enhance the antibody response. Ann. N.Y. Acad. Sci. **332**:564–578.
6. **Butler, R. C., A. Nowotny, and H. Friedman.** 1981. Induction of immunomodulatory factors by LPS and nontoxic derivatives, p. 181–198. *In* Immunomodulation by bacteria and their products. Plenum Publishing Corp., New York.
7. **Cunningham, A. J., and A. Szenberg.** 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology **14**:599–601.
8. **Friedman, H. F., and R. C. Butler.** 1980. Immunomodulatory effects of endotoxin-induced factors, p. 44–48. *In* D. Schlessinger (ed.), Microbiology—1980. American Society for Microbiology, Washington, D.C.
9. **Friedman, H. F., and W. S. Ceglowski.** 1971. Leukemia virus-induced alteration of leukocyte migration in vitro. Nature (London) **233**:415–416.
10. **Friedman, H. F., and W. S. Ceglowski.** 1971. Leukemia virus-induced immunosuppression. VIII. Rapid depression of in vitro leukocyte migration after infection of mice with Friend leukemia virus. J. Immunol. **107**:1673–1681.
11. **Hirano, S., H. Friedman, and W. S. Ceglowski.** 1971. Immunosuppression by leukemia viruses. VII. Stimulatory effects of Friend leukemia virus on pre-existing antibody-forming cells to sheep erythrocytes and *Escherichia coli* in nonimmunized mice. J. Immunol. **107**:1400–1409.
12. **Kamo, I., S.-H. Pan, and H. Friedman.** 1976. A simplified procedure for in vitro immunization of dispersed spleen cell cultures. J. Immunol. Methods **11**:55–62.
13. **Kately, J. R., I. Kamo, G. Kaplan, and H. Friedman.** 1974. Suppressive effect of leukemia virus-infected lymphoid cells on in vitro immunization of normal splenocytes. J. Natl. Cancer Inst. **53**:1371–1378.
14. **Notkins, A. L., S. E. Mergenhagen, and R. J. Howard.** 1970. Effect of virus infections on the function of the immune system. Annu. Rev. Microbiol. **24**:525–538.
15. **Nowotny, A., K. R. Cundy, N. L. Neale, A. M. Nowotny, P. Radvany, S. P. Thomas, and D. J. Tripodi.** 1966. Relation of structure to function in bacterial O-antigens. IV. Fractionation of the components. Ann. N.Y. Acad. Sci. **133**:586–603.
16. **Specter, S. C., M. Bendinelli, W. S. Ceglowski, and H. Friedman.** 1978. Macrophage induced reversal of immunosuppression by leukemia viruses. Fed. Proc. **37**:97–101.
17. **Specter, S., N. Patel, and H. Friedman.** 1976. Restoration of leukemia virus-suppressed immunocytes in vitro by peritoneal exudate cells. Proc. Soc. Exp. Biol. Med. **151**:163–167.