

ANALYSIS OF THE DEFECTS RESPONSIBLE FOR THE  
IMPAIRED REGULATION OF EPSTEIN-BARR VIRUS-INDUCED  
B CELL PROLIFERATION BY  
RHEUMATOID ARTHRITIS LYMPHOCYTES

I. Diminished Gamma Interferon Production  
in Response to Autologous Stimulation\*

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Normal adult T lymphocytes control the rate of Epstein-Barr virus (EBV)<sup>1</sup>-induced proliferation of B cells (1, 2). T cells of patients with rheumatoid arthritis (RA) do not regulate the in vitro EBV infection as efficiently, and EBV-induced B cell lines develop more rapidly from RA peripheral blood lymphocytes (3, 4). There is also evidence that activated T cells produce lymphokines capable of modulating EBV-induced lymphoblast transformation (5, 6). Therefore, we have sought to determine whether the defective regulation by RA T cells is associated with a difference in lymphokine production or activity.

Because mixed leukocyte reactions (MLR) generate considerable lymphokine activity, and because the autologous MLR appears to be involved in immunoregulatory mechanisms (7-11), supernatants from allogeneic and autologous MLR cultures were tested for their effects on EBV-induced B cell proliferation. We found that RA and normal T cells respond equally to allogeneic challenge with the production of a lymphokine that effectively controls the lymphoblast transformation. Autologous MLR supernatants from normal cells also effectively slow the rate of B cell proliferation, but RA T cells are specifically deficient in the production of this immunoregulatory lymphokine when stimulated by autologous non-T cells. The lymphokine has the physicochemical characteristics of human gamma interferon, its activity is mimicked by gamma interferon added to EBV-infected B cells, and the lymphokine activity is removed from culture supernatants reacted with antibodies specific for gamma interferon. Thus, the defective regulation of EBV-induced lymphoblast

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<sup>1</sup> Abbreviations used in this paper: AC, adherent cells; EBV, Epstein-Barr virus; FCS, fetal calf serum;  $\gamma$ -GFHS,  $\gamma$ -globulin-free horse serum; MLR, mixed leukocyte reaction; RA, rheumatoid arthritis; R-FCS, RPMI 1640 supplemented with FCS.

transformation by T cells from patients with RA may be due to diminished generation of gamma interferon by the T cells interacting with EBV-infected B cells.

### Materials and Methods

**Subjects.** Heparinized venous blood was obtained from 26 patients with definite or classical seropositive RA (12) between the ages of 25 and 70 yr (mean 56 yr); 12 patients with spondyloarthropathies (5 with ankylosing spondylitis, 3 with Reiter's syndrome, and 4 with psoriatic arthritis), mean age 43 yr (range 21–66 yr), all taking nonsteroidal anti-inflammatory agents; and 32 normal blood donors between the ages of 17 and 84 yr (mean 40 yr). All but one of the RA patients were taking nonsteroidal anti-inflammatory agents and, in addition, about one-half of them received either gold or D-penicillamine. Eight RA patients were also being treated with small doses (<10 mg/d) of prednisone.

**Cell Separation.** Peripheral blood mononuclear cells were obtained from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation, and T cells were separated from non-T cells by erythrocyte (E)-rosetting with neuraminidase-treated sheep erythrocytes as previously described (3). The non-T cell population was composed of 25–30% immunoglobulin-bearing cells as determined by direct immunofluorescence with F(ab')<sub>2</sub> goat anti-human immunoglobulins (N. L. Cappel Laboratories, Cochranville, PA), 50–60% esterase-positive cells (13), and <2% E-rosetting cells. The T cell populations contained <5% immunoglobulin-bearing cells. For adherent cell depletion, the non-T cells were resuspended at a density of  $1.0 \times 10^7$ /ml in RPMI plus 20% fetal calf serum (FCS) and plated in 2-ml aliquots into 60-mm-diam glass petri dishes. After incubating at 37°C for 1 h, the nonadherent cells are removed by aspiration and are further depleted of adherent cells by passage through G-10 Sephadex columns (14). The resultant adherent cell-depleted non-T population contained <1% esterase-positive cells and 50–70% surface immunoglobulin-bearing cells.

**Cytofluorographic Sorting.** Double-E-rosetted T cells are reacted with each monoclonal antibody (OKT4 and OKT8; Orthoclone, Ortho Pharmaceutical, Raritan, NJ) using 1  $\mu$ l of antibody plus 1  $\mu$ l of 10%  $\gamma$ -globulin-free horse serum ( $\gamma$ -GFHS)-RPMI (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) per  $1 \times 10^6$  cells. This cell suspension is incubated at 0°C for 30 min, then layered over 5 ml of cold  $\gamma$ -GFHS, pelleted at 400 g for 10 min, and washed twice with 10%  $\gamma$ -GFHS-RPMI. The cells to be sorted are resuspended in 2  $\mu$ l of rabbit anti-mouse IgG-fluorescein isothiocyanate (1:40 dilution) per  $1 \times 10^6$  cells, incubated in the dark at 0°C for 30 min, relayered over  $\gamma$ -GFHS, and pelleted at 400 g for 10 min. After two washes, the cells are resuspended at a density of  $2.5 \times 10^6$  cells/ml in RPMI-20% FCS. An aliquot of purified T cells ( $1-2 \times 10^6$  cells), stained in parallel with a nonreactive mouse myeloma protein of the same subclass as the monoclonal antibody (RPC5), provides control cells for establishing the gating settings on the cell sorter (Ortho model 50D with an argon laser). The yield of OKT8<sup>+</sup> cells has been  $0.5-2 \times 10^5$  cells/ml of blood with 94–98% purity (<1% OKT8<sup>+</sup> cells in the OKT8<sup>-</sup> population). For OKT4<sup>+</sup>, the yield has been  $2-4 \times 10^5$  cells/ml of blood with 95–99% purity (<<1% OKT4<sup>+</sup> cells in the OKT4<sup>-</sup> population).

**MLR Cultures.** Non-T cells were treated with 25  $\mu$ g/ml mitomycin C (Sigma Chemical Co., St. Louis, MO), for 60 min at 37°C, followed by three washes in RPMI-10% FCS.  $1 \times 10^5$  mitomycin-treated non-T cells were cultured with  $2 \times 10^5$  autologous or allogeneic T lymphocytes in 0.2 ml RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and penicillin-streptomycin at 5,000 U/ml (R-FCS) in flat-bottomed Linbro plates 76-001-05; Flow Laboratories, Hamden, CT. [<sup>3</sup>H]Thymidine incorporation [1  $\mu$ Ci/well; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NJ] was added during the last 18 h of incubation. The cultures were harvested on day 7 with a semi-automated multiple sample harvester (MASH) device. Data are expressed as the mean <sup>3</sup>H counts per minute of triplicate cultures.

**Preparation of Supernatants.** MLR-derived supernatants were produced by T cells that were stimulated by mitomycin C-treated non-T cells in 2 ml R-FCS at a responder-to-stimulator ratio of 2:1 and a total cell density of  $10^5$  cells/ml. After 72 h, the supernatants were recovered and filtered through 0.45- $\mu$ m membranes (Millipore Corp., Bedford, MA). The supernatants were stored at -80°C until used.

**Virus.** Transforming virus was obtained from the supernatants of the EBV-secreting B95-8 marmoset lymphoblastoid cell line (15), as described previously (3). The supernatant lympho-

cyte-transforming titer, calculated as described by Reed and Muench (16), was  $4.4 \log_{10}$  50% transforming doses/ml.

**EBV Interference Assay.** Cells were infected with EBV by adding 0.05 ml of B95-8 supernatant to  $1 \times 10^6$  double-rosetted non-T lymphocytes from normal donors in 1 ml R-FCS, followed by incubation at 37°C for 60 min in a humidified 5% CO<sub>2</sub>-in-air atmosphere. After three washes in R-FCS, the infected cells were cultured at  $5 \times 10^4$  cells/well in flat-bottomed, 96-well microtiter plates in 0.2 ml R-FCS alone or R-FCS mixed with MLR supernatant at 1:2 or 1:4 final dilutions. After 5–6 d, 75–100  $\mu$ l of medium was removed from the upper half of each culture and replaced with an equal volume of fresh complete medium.

EBV-induced cell proliferation was assessed by measuring [<sup>3</sup>H]thymidine incorporation on day 10 or as described in individual experiments. The data obtained are expressed as percent suppression which are calculated as percent suppression =  $1 - [(cpm \text{ in MLR supernatant-treated cultures}) / (cpm \text{ in control cultures})] \times 100$ . Factors produced by lymphocytes of the same individual in different experiments were similarly effective against EBV-infected lymphocytes from a panel of different cell donors. For example, auto-MLR supernatants from subject BF, generated on three different occasions, 12 wk apart, and tested in 10 different experiments with B lymphocytes from 7 different donors caused a mean suppression of  $67 \pm 3\%$  (SE).

**Interferon and Anti-Interferon Antibodies.** Human leukocyte alpha interferon standard (G-023-901-527), human fibroblast reference beta interferon (G-023-902-527), and sheep antisera to human alpha interferon standard (G-026-502-568) and to human beta interferon (G-028-501-568) were provided by the National Institute of Allergy and Infectious Diseases. Gamma interferon, prepared as previously described (17), was kindly provided by Dr. J. Vilcek, Department of Microbiology, New York University Medical Center, New York. Rabbit antiserum to human gamma interferon (18) and a different gamma interferon preparation were provided by Dr. G. J. Stanton, University of Texas Medical Branch, Galveston, TX. Mouse monoclonal antibody against human gamma interferon (19) was provided by Dr. H.-K. Hochkeppel, Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, Federal Republic of Germany, and a goat antibody against 45,000-mol wt human gamma interferon was provided by Dr. M. de Ley and Dr. A. Billiau, Rega Institute for Medical Research, University of Leuven, Leuven, Belgium (20).

**Interferon Assay.** A quantitative inhibition of cytopathic effect assay for human interferon, as described by Epstein et al. (21), was used with A-549 monolayer cells (derived from a carcinoma of the lung) and encephalomyocarditis virus as the challenge virus.

**Staphylococcus Bacteria Strain Cowan I Interference Assay.** Staphylococcus bacteria strain Cowan I, formaldehyde- and heat-killed, (Pansorbin; Calbiochem-Behring Corp., San Diego, CA) was added to double-rosetted non-T cells at concentrations of 0.001–0.01 vol/vol. B cells ( $5 \times 10^4$ – $1 \times 10^5$  cells/well) were cultured with Pansorbin with or without auto-MLR supernatants at 1:2 or 1:4 final dilutions. DNA synthesis was assessed on day 4 by measuring [<sup>3</sup>H]thymidine incorporation. The data obtained are expressed as percent suppression, and are calculated as described above.

## Results

During the autologous MLR, cells from healthy donors release a factor(s) into the culture medium that retards the rate of EBV-induced B lymphoblast out-growth in a dose-related manner (Fig. 1). EBV-induced B cell proliferation, measured 10 d after virus infection, was inhibited  $50 \pm 3\%$  (mean  $\pm$  SE) by dilutions of 72-h auto-MLR supernatants generated by cells from 32 normal donors. Studies of the time course of factor generation revealed that maximum suppression was observed in supernatants obtained after 72 h in the auto-MLR. Proliferation was inhibited 15% by 24-h supernatants, 25% by 48-h supernatants, and 48% by 72-h supernatants from auto-MLR generated by cells from four normal donors. Longer culturing of the auto-MLR through 6 d did not yield more inhibitory activity.

In contrast to the inhibitory activity generated by cells from normal donors, RA auto-MLR supernatants were ineffective regulators; 72-h supernatants from 26 RA

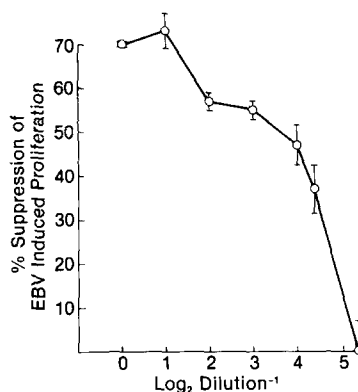


FIG. 1. Suppression of EBV-induced B cell proliferation by dilutions of a 72-h supernatant from a normal auto-MLR. Results are expressed as the mean percent suppression of [<sup>3</sup>H]thymidine incorporation compared with control cultures 10 d post-EBV infection (mean control counts = 59,109). Error bars indicate the SEM of quadruplicate cultures.

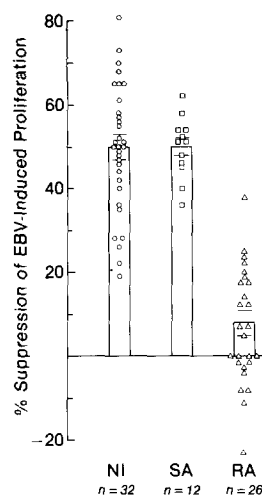


FIG. 2. Effect of autologous MLR-derived supernatants from normal subjects (NI), patients with spondyloarthropathies (SA), and patients with rheumatoid arthritis (RA) on EBV-induced B cell proliferation. Results are expressed as the mean percent suppression of the [<sup>3</sup>H]thymidine incorporation compared with control cultures at 10 d post-EBV infection (mean control counts from 32 experiments = 50,181 cpm). Each point represents the average of the results of three separate experiments for each subject done at different times. Error bars represent the mean  $\pm$  SEM for all individuals in the designated groups.

patients inhibited EBV-induced B cell proliferation only  $8 \pm 3\%$  (Fig. 2). The inhibitory activity did not increase in RA supernatants obtained within up to 5 d of auto-MLR. Diminished lymphokine activity from RA cells could not be attributed to the drugs taken by the patients. Auto-MLR supernatants from 12 subjects with active spondyloarthropathies receiving similar nonsteroidal anti-inflammatory drugs inhibited EBV-induced DNA synthesis ( $50 \pm 2\%$ ) as well as those from normal donors (Fig. 2). The lack of inhibitory activity in auto-MLR supernatants was observed in samples from 18 RA patients who were not treated with corticosteroids ( $6 \pm 3\%$ ), as well as those from 8 RA patients taking prednisone at  $\leq 10$  mg/ $(12 \pm 5\%)$ . Similarly,

there was no difference between the 14 patients receiving gold or D-penicillamine ( $8 \pm 3\%$ ) and the 12 RA patients not taking those drugs ( $8 \pm 4\%$ ). Although there is a modest decrease in inhibitory activity in auto-MLR supernatants from older subjects ( $43 \pm 3\%$  mean suppression in supernatants from 15 donors over age 40 compared with  $52 \pm 2\%$  suppression in those from donors younger than 40), age cannot account for the striking differences between RA and normal supernatants.

Supernatants of activated lymphocytes are known to contain a variety of lymphokines, some with stimulatory capacities and others suppressive. We considered the possibility that a difference in the balance between helper and suppressor lymphokines or the generation of inhibitors of the suppressive lymphokines might explain the discrepancy observed when RA and normal T cells were stimulated by autologous mononuclear cells. However, mixing nonsuppressive RA-cell-produced MLR supernatants with suppressive supernatants from normal cells failed to demonstrate any signs of competition (Fig. 3).

The inhibitory factor(s) in the auto-MLR supernatants exerts its effects relatively

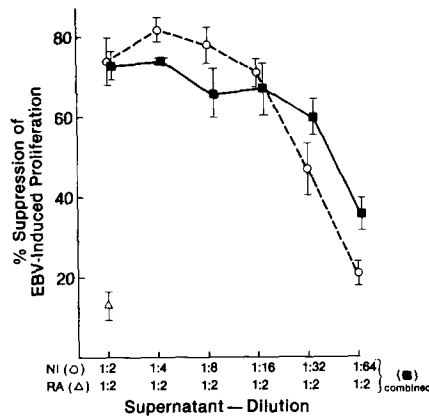


FIG. 3. The effect of mixing RA-cell-derived auto-MLR supernatants with supernatants from normal auto-MLR on the suppression of EBV-induced B cell proliferation. Dilutions of a normal supernatant were added to EBV-infected non-T cells alone or in the presence of a constant 1:2 dilution of an RA supernatant. Results are expressed as the mean percent suppression of [ $^3\text{H}$ ] thymidine incorporation compared with control cultures 10 d post-EBV infection (mean control counts = 32,543). Error bars indicate the SEM of quadruplicate cultures.

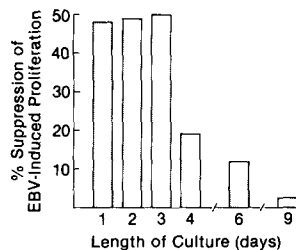


FIG. 4. The effect of a delayed addition of a supernatant from a normal auto-MLR on the suppression of EBV-induced B cell proliferation. Supernatants were added at the times indicated after EBV infection of normal non-T lymphocytes. Results are expressed as the mean percent suppression of [ $^3\text{H}$ ]thymidine incorporation compared with control cultures 10 d post-EBV infection (mean control counts = 53,385 cpm). Error bars indicate the SEM of quadruplicate cultures.

TABLE I  
*Identification of the T Cells Required for the Generation of Supernatant-mediated Inhibition of EBV-induced B Cell Proliferation*

Auto-MLR*		Supernatant‡	
Responder	Stimulator	Percent suppression	Interferon¶ U/ml
E-RFC	-	17 ± 3	NT
	+	76 ± 4	37
OKT4 <sup>+</sup>	-	4 ± 12	NT
	+	67 ± 3	23
OKT8 <sup>+</sup>	-	0 ± 3	NT
	+	18 ± 8	<1
Non-T	-	7 ± 2	NT

\* Auto-MLR were established with 10<sup>6</sup> responder cells/ml with (+) or without (-) 5 × 10<sup>5</sup> mitomycin-treated non-T stimulator cells/ml.

‡ Auto-MLR supernatants were obtained after 72 h of culture.

§ E-RFC = twice-E-rosetted T cells; OKT4<sup>+</sup> and OKT8<sup>+</sup> cells were obtained with the fluorescence-activated cell sorter as described in Materials and Methods. Non-T indicates peripheral blood mononuclear cells depleted of E-rosetting cells.

|| Results are expressed as the mean ± SEM % suppression of EBV-induced B cell proliferation compared with control cultures at 10 d post-EBV infection (mean control counts = 89,990 com).

¶ Data is expressed as the mean of two determinations of interferon activity assayed as described in Materials and Methods. Units expressed as equivalents to the antiviral effect of the international standard for alpha interferon.

early after EBV infection. The susceptibility of the infected cells to inhibition by supernatants is maximal immediately after infection and persists for 3 d. Addition of supernatants ≥96 h after EBV infection had little or no effect on subsequent lymphoblast proliferation (Fig. 4). Similarly, auto-MLR supernatants had no significant effect on the proliferation of previously established permanent B lymphoblastoid cell lines. Thus, the inhibition of EBV-induced B cell proliferation does not appear to be due to direct inhibition of DNA synthesis or other biochemical events required for cell proliferation.

The inhibitory potency of the auto-MLR supernatants is not related to the DNA synthetic response of the T cells to autologous non-T cell stimulation. The correlation coefficients obtained when comparing [<sup>3</sup>H]thymidine incorporation on day 7 of the auto-MLR with the inhibitory activity of day 3 supernatants were  $r = 0.31$  in samples from normal subjects and  $r = 0.11$  in cultures from RA patients. In addition, the mean DNA synthetic response of RA cells in the auto-MLR, 11,359 cpm ± 2,329 SE, and the mean response in normal cell populations, 13,535 cpm ± 2,102 SE did not differ significantly. Finally, the generation of the inhibitory factor in the auto-MLR does not require T cell proliferation. Pretreatment of the responder T cells with mitomycin C at a concentration that inhibited auto-MLR-induced DNA synthesis >90% and X irradiation of the T cells with 2,000 rad, eliminating their DNA synthetic response to autologous cell stimulation, did not interfere with factor elaboration.

The types of T cells required for the generation of the inhibitory factor was analyzed by separating T cells from normal donors into OKT4<sup>+</sup>- and OKT8<sup>+</sup>-enriched subpopulations using a fluorescence-activated cell sorter. When OKT4<sup>+</sup> cells were

used as responders in the auto-MLR, the supernatant inhibitory activity was essentially the same as that obtained with the unfractionated T cells (Table I). The OKT8<sup>+</sup> cells generated little suppressive activity in response to autologous non-T cells.

Allogeneic MLR also generate a factor(s) that suppresses EBV-induced lymphoblast outgrowth. In fact, the inhibitory activity was stronger in allo-MLR-derived supernatants than in those generated in the auto-MLR. However, unlike the auto-MLR responses, cells from RA patients produce as much inhibitory activity on allogeneic stimulation as do normal T cells (Fig. 5 A). When serially diluted, the RA and normal T cell supernatants lost their suppressive activities in parallel. The most potent supernatants retained their activity to dilutions of up to 1:200. The inhibitory activity generated in the auto- and allo-MLR have similar physicochemical properties. They are heat labile (heating to 56°C for 30 min removed ~60% of the effect, whereas after 60 min all inhibitory activity was lost), and are inactivated at pH 2.0 (Fig. 6). Chromatography on Sephadex G-100 revealed that the active factor has a molecular weight of ~50,000 (Fig. 7).

Because RA T cells produce inhibitory factors when challenged in the MLR by allogeneic cells but not autologous cells, we examined the autologous non-T cell stimulator population for its contribution to the RA defect. 72-h auto-MLR supernatants from intact and adherent cell (AC)-depleted cultures, diluted 1:2, were tested for their effects on DNA synthesis in B cells 10 d after virus infection (Fig. 8). The inhibitory activity in supernatants produced by cells from nine normal donors did not change significantly after AC depletion ( $58 \pm 4\%$  SE before and  $65 \pm 7\%$  after AC depletion). In supernatants from eight RA patients, by contrast, inhibition of EBV-induced DNA synthesis increased from  $11 \pm 6$  to  $52 \pm 6\%$  after monocyte depletion. Thus, RA cells are able to respond to autologous stimulation with the generation of a factor that can inhibit EBV-induced B cell proliferation, but their capacity to do so is diminished in the presence of autologous AC.

We have examined the relationship between the inhibitory lymphokines and

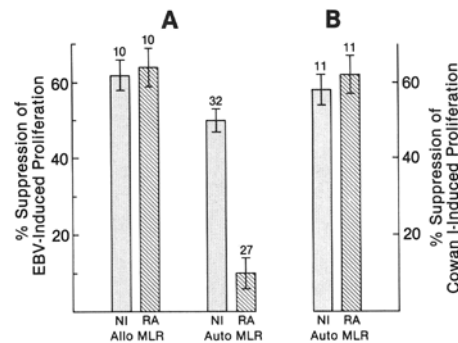


FIG. 5. (A) Suppression of EBV-induced B cell proliferation by the addition of 1:2 dilutions of auto-MLR- and allo-MLR-derived supernatants from RA and normal (N) subjects. Results are expressed as the mean percent suppression of [<sup>3</sup>H]thymidine incorporation compared with control cultures at 10 d post-EBV infection. Error bars indicate the SEM of quadruplicate cultures. The numbers of subjects tested in each group are shown above the bars. (B) Suppression of Staphylococcus bacteria strain Cowan I-induced B cell proliferation by the addition of 1:2 dilutions of auto-MLR-derived supernatants from RA and normal subjects (mean control counts of =52,214 cpm). Error bars represent the SEM of quadruplicate cultures. The numbers of subjects tested in each group are shown above the bars.

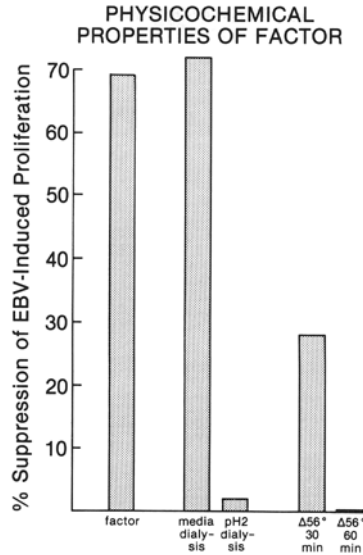


FIG. 6. Physicochemical properties of the inhibitory factor in auto-MLR supernatants. The supernatant was dialyzed for 24 h at 4°C against RPMI 1640 with multiple changes of the medium. An acid-treated supernatant was prepared by dialysis at 4°C for 18 h against pH 2 glycine buffer followed by an additional 18 h dialysis against RPMI 1640 to remove excess acid. Thermal stability of the inhibitory activity was evaluated by heating the supernatants in a water bath at 56°C for 30 and 60 min. The treated supernatants were tested for suppression of EBV-induced B cell proliferation. The data are expressed as the mean percent suppression of [<sup>3</sup>H]thymidine incorporation compared with control cultures at 10 d post-EBV infection. Treatment of allo-MLR supernatants produced the same results.

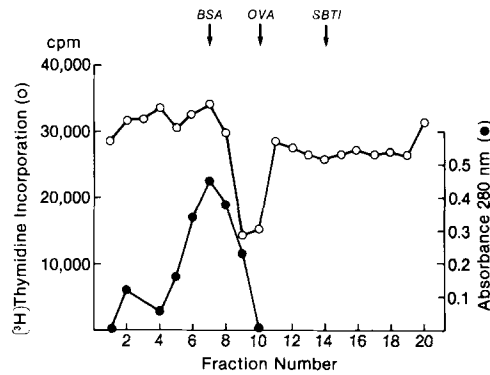


FIG. 7. The ability of Sephadex G-100 fractions of a supernatant from normal auto-MLR to suppress EBV-induced B cell proliferation. Molecular-weight markers included bovine serum albumin (68,000 mol wt), ovalbumin (44,000 mol wt), and soybean trypsin inhibitor (21,500 mol wt). [<sup>3</sup>H]Thymidine incorporation was measured 10 d post-EBV infection. Results are expressed as the mean counts per minute of triplicate cultures.

interferon. Interferon was measured in 18 MLR supernatants chosen because they gave various amounts of suppression of EBV-induced B cell proliferation. The supernatants were produced by RA and normal cells in autologous and allogeneic MLR. Their range of suppression was 0–90%, and interferon levels varied from undetectable to 37 U/ml (units are determined as equivalents to the antiviral effect



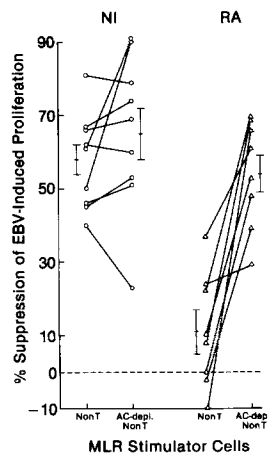


FIG. 8. The effect of removal of AC from mononuclear cell populations on the production of a supernatant inhibitory factor in auto-MLR. Supernatants collected at 72 h were tested for their ability to suppress EBV-induced B cell proliferation. Normal ( $\circ$ ) and RA ( $\Delta$ ) T cells were stimulated with autologous non-T cells before and after removal of AC by passage through Sephadex G-10 columns. Results are expressed as the mean percent suppression of [ $^3$ H]thymidine incorporation compared with control cultures at 10 d post-EBV infection (mean control counts = 69,055 cpm).

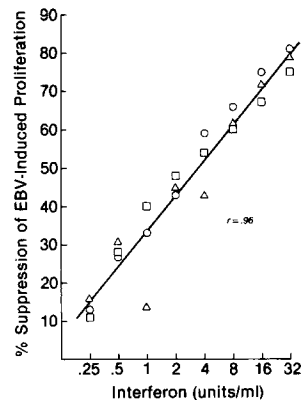


FIG. 9. The effect of NIH standard IFN ( $\circ$ ), IFNB ( $\Delta$ ), IFN $\gamma$  ( $\square$ ) on EBV-induced B cell proliferation. Interferon preparations were added in concentrations indicated to EBV-infected normal non-T lymphocytes. Results are expressed as the mean percent suppression of [ $^3$ H]thymidine incorporation of quadruplicate samples compared with the control culture at 10 d post-EBV infection (mean control counts = 79,169 cpm). The correlation coefficient ( $r = 0.96$ ) was calculated using  $\log_2$  of the interferon concentrations.

of the international standard for alpha interferon). There was a significant correlation between the suppressive activity of the individual supernatant and its interferon content ( $r = 0.63$ ;  $P < 0.01$ ). Interferon was below the level of detection in all of the tested RA auto-MLR supernatants. If the inhibitory activity we are measuring is mediated by interferon, then addition of small amounts of purified interferon to EBV-infected B cells should mimic the inhibitory action of auto-MLR supernatants. In fact, human alpha and beta interferons (National Institutes of Health standards) and two preparations of human gamma interferon caused a 50% suppression of EBV-induced B cell proliferation with  $\sim 2$ – $3$  interferon U/ml (Fig. 9).

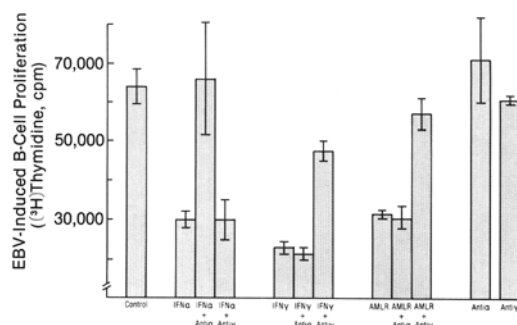


FIG. 10. The effect of antibodies to various interferons on the inhibition of EBV-induced B cell proliferation by human alpha interferon (5 IU/ml); human gamma interferon (5 IU/ml); and a normal auto-MLR supernatant diluted 1:2. The inhibitory factors (untreated or after a 60-min preincubation with the antiserum indicated) were added to EBV-infected non-T cells. [<sup>3</sup>H]Thymidine incorporation was measured at 10 d post-EBV infection. Results are indicated as the SEM of quadruplicate culture.

TABLE II

*Neutralization of Auto-MLR Supernatant-mediated Inhibition of EBV-induced B Cell Proliferation by Monoclonal Antibody to Human Gamma Interferon\**

Supernatant	Antibody dilution	[ <sup>3</sup> H]thymidine incorporation‡	Percent suppression§
		<i>mean cpm ± SEM</i>	
Medium	—	59,824 ± 3,136	—
	1:30,000	52,717 ± 3,132	—
	1:3,000	61,312 ± 2,602	—
	1:300	53,783 ± 3,732	—
Auto-MLR, 1:2	—	33,587 ± 508	44
	1:30,000	39,404 ± 1,332	25
	1:3,000	61,031 ± 4,251	1
	1:300	59,647 ± 5,218	0
Auto-MLR, 1:8	—	44,773 ± 1,012	25
	1:30,000	55,020 ± 2,142	0
	1:3,000	64,177 ± 5,432	0
	1:300	62,277 ± 1,222	0

\* Fresh medium or auto-MLR supernatant diluted 1:2 or 1:8 was preincubated with murine IgM monoclonal antibody to human gamma interferon at the dilutions indicated and then added to EBV-infected non-T cells as described in Materials and Methods.

‡ [<sup>3</sup>H]Thymidine incorporation was assessed 10 d post-EBV infection and the data expressed as the mean ± SEM of quadruplicate cultures.

§ Data is expressed as percent suppression as compared with control cultures treated with some antibody dilution calculated as described in Materials and Methods.

The physical properties of the inhibitory factor in the auto-MLR supernatants are similar to those of gamma interferon. To determine whether gamma interferon is the active factor, the supernatants were reacted with antibodies to human interferons. The inhibitory factor's action was blocked by antiserum to gamma interferon but not by standard NIH antisera to alpha interferon (Fig. 10) or beta interferon (data not shown). Although the antiserum against alpha interferon had no effect on the inhibitory activity of the auto-MLR supernatant, it completely neutralized the

suppressive activity of leukocyte interferon. The auto-MLR inhibitory lymphokine activity was also blocked by a murine IgM monoclonal antibody against human gamma interferon in a dose-related fashion (Table II). Control IgM ascites had no effect on the inhibitory activity of MLR supernatants. In addition, the anti-viral effect of the auto-MLR supernatants in the interferon assay was completely neutralized with goat anti-human gamma interferon but not affected by sheep anti-human alpha interferon (data not shown). Anti-interferon preparations had no direct effect on EBV-induced B cell proliferation. Thus, the physicochemical properties, functional characteristics, and reactivity with specific polyclonal and monoclonal antibodies identify this MLR-derived lymphokine as human gamma interferon.

To determine whether the effects of gamma interferon on EBV-induced B cell proliferation might reflect a virus-specific event or a more general effect on B cell activation, auto-MLR supernatants generated by normal or RA cells were tested for inhibitory activity to B cells stimulated by another T cell-independent activator, *Staphylococcus* bacteria strain Cowan I (Fig. 5 B). Auto-MLR supernatants from 11 normal blood donors and from 11 RA subjects were equally suppressive to Cowan I-induced B cell proliferation (normal  $58 \pm 4\%$ ; RA  $62 \pm 5\%$ ). Therefore, RA cells can generate other potentially immunoregulatory suppressor factors in response to autologous lymphoid cell stimulation, although they do not produce gamma interferon. The factor(s) involved in the regulation of Cowan I-induced B cell proliferation has distinct physicochemical characteristics from gamma interferon, it is stable to  $56^{\circ}\text{C}$  treatment, and its activity is lost after dialysis.

### Discussion

The results of these experiments lead to two conclusions of interest: (a) normal T cells activated in autologous and allogeneic MLR produce sufficient amounts of gamma interferon to inhibit *in vitro* EBV-induced B cell proliferation; and (b) RA T cells are specifically deficient in their ability to generate gamma interferon when stimulated by autologous cells, but are capable of doing so, and do so normally, when stimulated by allogeneic cells. The inhibitory effect of gamma interferon parallels the characteristics of T cell-mediated early-phase regulation of EBV-induced B lymphoblast transformation (1, 3, 4).

Using a microassay with cells that are relatively sensitive to gamma interferon (22), we detected small amounts of interferon activity (4–27 U/ml) in supernatants from most normal auto-MLR collected at 72 h. There was a significant correlation between this activity and the inhibition of EBV-induced B cell proliferation. The RA auto-MLR supernatants lacked both EBV proliferation inhibitors and interferon activity. The inhibition of EBV-induced B cell proliferation is itself a sensitive and reproducible interferon assay; 2–3 U/ml of either alpha, beta, or gamma interferon reduces proliferation ~50% and this effect is abrogated by the corresponding type-specific antibody to interferon.

Thorley-Lawson (23) demonstrated that T cells from normal adults are able to delay outgrowth of EBV-infected B cells and to suppress the EB nuclear antigen expression, if the interactions with the EBV-infected cell occurs within 24 h post-infection. More recent data suggest that this early suppressive event can be mediated by a lymphokine with properties of human leukocyte alpha interferon (5). We confirm that alpha interferon inhibits EBV-induced B cell proliferation. However, Thorley-

Lawson had to add ~1,000 U of alpha interferon to achieve 50% inhibition and 1,000 U of antibody added to that interferon was needed to block his supernatant activity. Our EBV-induced B cell proliferation assay required only 1–3 U of alpha interferon for 50% inhibition. The inhibitory activity in our auto- and allo-MLR supernatants differs from leukocyte interferon in its molecular weight, heat lability, and sensitivity to acid at pH 2. It also differs somewhat in the kinetics of its activity, retaining the capacity to inhibit EBV-induced proliferation up to 72 h after viral infection. The reason for these discrepancies may relate to Thorley-Lawson's use of EBV-infected cells to provoke interferon production where we used normal non-T cells as stimulators. The requirement for prior exposure to EBV in the responder T cells (5) supports a role for EBV antigens and suggests that virus-related membrane antigens such as lymphocyte-defined membrane antigen could be responsible for stimulating alpha interferon production.

The physicochemical properties of the auto-MLR supernatant factor are like undenatured human gamma interferon, which is known to be released into culture supernatants during allogeneic MLR, but has not been found when the stimulus is autologous lymphocytes (24). Two separate gamma interferon preparations (17, 18) were tested for inhibitory activity; both preparations were able to suppress EBV-induced B cell proliferation 50% at a concentration of ~2–3 interferon U/ml. We found that interferons alpha and beta also effectively inhibit EBV-induced B cell proliferation, but antisera to those interferons do not block the inhibitory activity in auto-MLR supernatants. In contrast, antiserum to human gamma interferon eliminates the auto-MLR-derived inhibitory activity; and this finding was confirmed with a monoclonal antibody against human gamma interferon (19). Antiviral activity was also removed from the auto-MLR supernatants by treatment with antiserum to human gamma interferon. Taken together, these experiments identify the regulatory factor in autologous and allogeneic MLR supernatants that inhibits EBV-induced B cell proliferation as human gamma interferon. T cells from patients with rheumatoid arthritis are defective in their ability to generate gamma interferon during the autologous MLR, which may explain, at least in part, the diminished capacity of RA T cells to control EBV-induced B lymphoblast transformation (3, 4).

RA T cells can make gamma interferon and do so in normal amounts when stimulated by allogeneic cells. Thus, the defect in RA appears to reside in the unique cell-cell interactions that characterize the autologous MLR. Surprisingly, the proliferative response induced by the auto-MLR does not correlate with the amounts of factor produced. The mean DNA synthetic response of RA cells in the auto-MLR was not significantly different from the mean response in normal cell populations, nor was there a correlation between the auto-MLR proliferative response on day 7 and the gamma interferon activity of day 3 supernatants in RA patients or normal blood donors. Most striking was the finding that adherent cell depletion of the RA non-T cells led to a marked increase in gamma interferon production while the proliferative response fell sharply.

Diminished gamma interferon generation by RA cells cannot be explained by the effects of either chronic inflammatory disease or drug therapy. Cells from patients with active spondyloarthritides, all of whom had chronic joint disease of similar severity and duration as the RA patients and who were being treated with nonsteroidal anti-inflammatory medications, produced gamma interferon in the auto-MLR as

effectively as cells from healthy donors. Furthermore, cells from RA subjects receiving corticosteroids, gold, or D-penicillamine did not differ from others with RA in their inability to generate gamma interferon in the auto-MLR. None of the patients used in these studies were receiving other immunosuppressive drugs.

Supernatants of activated lymphocytes are known to contain a variety of lymphokines, some with stimulatory capacities and others suppressive (reviewed in 25, 26). We considered the possibility that a difference in the balance between these effects might explain the discrepancy observed when RA and normal T cells were stimulated by autologous mononuclear cells. However, mixing nonsuppressive RA cell-produced MLR supernatants with inhibitory supernatants from normal cells failed to demonstrate any signs of competition. Thus, the RA cells do not appear to be producing a disproportionate amount of a "helper" factor that overwhelms the suppressor; nor are they producing an inhibitor of gamma interferon.

Interferons have growth inhibitory as well as anti-viral properties. We were interested in determining whether the striking abnormality in the RA auto-MLR supernatant factors was limited to EBV-induced lymphoblast transformation or reflected a broader defect in control of cell proliferation. Because EBV activates B cells directly without requiring the participation of T cells (27, 28), we studied the effects of the RA auto-MLR supernatants on B cell proliferation induced by *Staphylococcus* bacteria strain Cowan I, which is also a relatively T cell-independent response (29, 30). In marked contrast with the results obtained with EBV as the B cell stimulant, autologous MLR supernatants from RA patients contain potent inhibitors of Cowan I-induced B cell proliferation, equivalent to normal control supernatants. We have not yet characterized the material responsible for inhibiting Cowan I-induced B cell proliferation, but its physicochemical characteristics differ from gamma interferon.

Cultured EBV-infected B lymphocytes become immunoglobulin-secreting cells. Tosato et al. (31) have demonstrated that RA T cells cannot suppress autologous EBV-induced immunoglobulin-secreting cells, but can regulate their allogeneic counterparts, which they have attributed to a specific regulatory RA T cell defect. The regulator RA T cells that we have studied are present and can respond normally to autologous B cells. The regulatory defect depends on the RA T cells interacting with autologous adherent cells. Our more recent results<sup>2</sup> demonstrate that the adherent cell effects are prostaglandin mediated and the regulatory defect reflects an enhanced sensitivity of RA T cells (or a T cell subset) to prostaglandin.

### Summary

T cells of patients with rheumatoid arthritis (RA) do not control the rate of B lymphoblast transformation induced by Epstein-Barr virus (EBV) as efficiently as T cells from healthy individuals; thus, lymphoblast cell lines are established more readily in RA lymphocytes in vitro after EBV infection. In the present experiments, we have asked whether this T cell regulation can be reproduced by lymphokines. We found that normal T cells, activated in allogeneic or autologous mixed leukocyte reactions (MLR), produce lymphokines that inhibit in vitro EBV-induced B cell

<sup>2</sup> Hasler, F., H. G. Bluestein, N. J. Zvaifler, and L. B. Epstein. Analysis of the defects responsible for the impaired regulation of EBV-induced B-cell proliferation by rheumatoid arthritis lymphocytes. II. Role of monocytes and the increased sensitivity of rheumatoid arthritis lymphocytes to prostaglandin E. Manuscript submitted for publication.

proliferation. Allogeneic MLR supernatants inhibited EBV-induced DNA synthesis  $62 \pm 4\%$  (mean  $\pm$  SE) at 10 d post-infection, whereas autologous MLR supernatants suppressed it  $50 \pm 3\%$ . RA T cell supernatants produced in an allogeneic MLR suppressed as well as normal T cell supernatants ( $64 \pm 5\%$  inhibition). In contrast, supernatants from RA autologous MLR had little inhibitory activity. EBV-induced DNA synthesis at 10 d was reduced only  $8 \pm 3\%$ , compared with the  $50 \pm 3\%$  suppressive activity of normal autologous MLR supernatants. The magnitude of the proliferative responses in the autologous MLR generating the lymphokines was similar in the normal and RA populations. After depletion of adherent cells from the RA auto-MLR stimulators, supernatant inhibitory activities increased to normal levels (from  $11 \pm 6$  [SE] to  $52 \pm 6\%$  [SE]).

The inhibitory factor involved in the regulation of *in vitro* EBV infection is a protein with a molecular weight of  $\sim 50,000$ . Its activity is eliminated by heating at  $56^\circ\text{C}$  and by exposure to acid at pH 2. The inhibitory activity is blocked by mixing the MLR supernatants with a polyvalent antisera or monoclonal antibodies specific for human gamma interferon. Gamma interferon produced by activating T cells in allo- or auto-MLR can reproduce T cell-mediated regulation of EBV-induced B cell proliferation, and the failure of RA auto-MLR to generate that lymphokine parallels the defective T cell regulation of EBV-induced B cell proliferation characteristic of RA lymphoid cells.

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