OBLIGATORY ROLE OF GAMMA INTERFERON IN T CELL-REPLACING FACTOR–DEPENDENT, ANTIGEN-SPECIFIC MURINE B CELL RESPONSES

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Antibody responses can be induced in vitro from B cell populations with a variety of stimuli, provided that T cells or various cytokines are present in the cultures. In most studies, initial signals for B cell activation are generated via immunoglobulin (Ig) receptors and are mediated by antigens or by anti-Ig antibodies (1–4). Subsequent B cell proliferation and differentiation to antibody-secreting cells is greatly augmented by the presence of soluble mediators generated in cultures containing activated T cells. Early studies described a T cell–replacing factor (TRF),[1] present in supernatants (Sn) of murine mixed leukocyte reactions (MLR), that supported antibody responses of spleen cells from nu/nu mice to sheep red blood cells (SRBC) (5). Subsequent work revealed a similar activity in Sn of concanavalin A (Con A)-stimulated spleen cells that was not active on cells from mice with the *xid* defect (6). Similar TRF activity for murine B cells has been shown with human MLR Sn (7), suggesting that the mediators can also function across species barriers.

Early efforts to characterize the molecular components of TRF met with little success, possibly because several mediators cooperated in the biological assays. Recent work has attempted to analyze the specific cytokines using Sn of monoclonal or long-term T cell lines and lymphokine fractions of known biological activity. Results from these studies (8–10) show that at least two different lymphokine preparations act synergistically to produce a TRF effect on B cells in plaque-forming cell (PFC) responses driven either by antigen or anti-Ig. One of the complementing components is usually obtained from T cell hybridomas, and contains interleukin 2 (IL-2). The other is obtained in Sn of a long-term alloreactive T cell line or some T-T hybridomas, and contains several biological activities, including γ interferon (IFN-γ). It is termed T cell–replacing factor

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[1] *Abbreviations used in this paper:* ATS, antithymocyte serum; BCGF, B cell growth factor; BRBC, burro red blood cells; C′, complement; Con A, concanavalin A; Hu IFN-γ, human IFN-γ; IFN-γ, γ interferon; IL-1, -2, and -X, interleukin 1, -2, and -X; MAF, macrophage-activating factor; MLR, mixed leukocyte reaction; Mu IFN-γ, murine IFN-γ; NK, natural killer; PFC, plaque-forming cell; RAM-IFN-γ, rabbit antiserum to mouse IFN-γ; RAH-IFN-γ, rabbit antiserum to human IFN-γ; rIFN-γ, recombinant-derived IFN-γ; SRBC, sheep red blood cells; Sn, supernatant; TRF, T cell–replacing factor.

(TRF) or interleukin X (IL-X) (11) and is active in responses to hapten-carrier antigens (12) as well as thymus-independent antigens (13). It is possible, however, that both Sn contain other mediators which may contribute to the antibody responses; some studies indicate that interleukin 1 (IL-1) (11) and perhaps other factors (10) may be required to obtain maximum TRF activity. However, the precise role for any of these factors in the antibody response is not known and the target cells for their actions remain to be identified.

Results from some recent studies have suggested that IFN-γ may be a component of TRF. Indirect evidence was obtained from the acid-labile character of TRF present in antigen- or Con A–stimulated T cell Sn (1, 12) and also from a correlation of IFN-γ levels and TRF activity of Sn of T cell hybridomas (14). This view is supported by recent data (15) showing that recombinant-derived murine IFN-γ (Mu rIFN-γ) can act as the second component of a complementing TRF system to support PFC responses to antigen (15). However, the specificity of the IFN-γ effect is unclear since it may also act as a polyclonal activator for B cell PFC responses (16). Together, the data suggest that purified IFN-γ may modulate B cells in their maturation to antibody production, but it is not clear whether this is accomplished through the pathway normally regulated by TRF. Similarly, it is possible that Con A or MLR Sn contain other TRF mediators, distinct from IFN-γ, which could independently support PFC responses.

In the present study, we have tested whether IFN-γ is essential in the TRF activity of four independent TRF preparations, including two derived from primate cells. We added to the cultures an antibody to IFN-γ that has been shown (17, 18) to neutralize the biological activity of IFN-γ in antiviral assays and macrophage activation assays; similar inhibitory effects have also been obtained in Ia antigen induction systems (19). The results clearly show that IFN-γ is a required mediator in the action of several forms of TRF, that it only acts relatively early in the response, and that, in cases of TRF which lack this factor, the IFN-γ is produced by the responding cells, despite extensive T cell depletion. The source of the IFN-γ may be the natural killer (NK) cells produced in these cultures.

Materials and Methods

Mice. (DBA/2 × CBA/N)F_1 and the reciprocal hybrid mice were purchased from Dominion Laboratories, Dublin, VA and outbred nude mice (nu/nu) were purchased from Charles River Breeding Laboratories, Wilmington, MA. CBA/J mice were purchased from The Jackson Laboratory, Bar Harbor, ME and BALB/c mice from Cumberland View Farms, Clinton, TN. All mice were 5–12 wk old when used.

Antigens and Other Reagents. SRBC were obtained from a single animal (B1) from the Navy Medical Research Institute, Bethesda, MD; burro red blood cells (BRBC) were purchased from Colorado Serum Co., Denver, CO. Con A was purchased from Difco Laboratories, Inc., Detroit, MI and alpha-methyl-D-mannoside from Sigma Chemical Co., St. Louis, MO. A human lymphokine-rich fraction, termed purified IL-2, was purchased from Electro-Nucleonics, Inc., Silver Spring, MD [TRF(E-N)]. B cells were prepared in RPMI 1640 supplemented with 2 ml L-glutamine, 25 mM Hepes, 50 μg/ml gentamycin sulfate, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Tissue Culture Biologicals, Tulane, CA). The medium for cell cultures also contained 5×10^{-5} M 2-mercaptoethanol and fetal calf serum from a selected lot (Biofluids Inc., Rockville, MD). Mu rIFN-γ and recombinant-derived human

IFN-γ (Hu rIFN-γ) were kind gifts of Dr. C. Sevastopolous, Genentech, Inc., South San Francisco.

Murine TRF. Murine TRF was prepared as described (5). Spleen cells from CBA/J and BALB/c mice were mixed in equal numbers to produce a final concentration of 10^7 cells/ml. The cells were cultured for 24 h and cell-free Sn were collected and stored at −80°C. A Con A–stimulated Sn was prepared as described (8). Briefly, spleen cells from (DBA/2 × CBA/N)F_1 mice were cultured at 10^7 cells/ml in the presence of 4 μg/ml of Con A. After 24 h, cell-free Sn were collected and stored at −80°C. When Con A Sn was used, 10 mg/ml of alpha-methyl-D-mannoside was added to all cultures.

Antisera. Antithymocyte serum (ATS) was prepared from rabbits hyperimmunized with mouse thymocytes; IgM monoclonal antibody to Thy-1.2 (clone F7D5) was used at a final concentration of 1:1000; rabbit anti-mouse IFN-γ (RAM-IFN-γ) (rabbit MI-3) and rabbit anti-human IFN-γ (RAH-IFN-γ) (rabbit 12R1) were prepared exactly as described by Svedersky et al. (18). Briefly, rabbits were immunized with rIFN-γ expressed in *Escherichia coli* that had been purified to the extent described earlier (20, 21), in complete Freund's adjuvant (200 μg protein in six subcutaneous sites). Afer 10 d, the same dose was given intramuscularly in incomplete Freund's adjuvant; after 20 d, 200 μg protein was given intravenously. On day 24 the rabbit was bled. The affinity-purified RAM-IFN-γ was the same preparation used by Svedersky et al. (18) and its preparation was described. Briefly, 12 mg of highly purified Mu rIFN-γ were coupled to Affi-gel 10 resin; 2 ml of hyperimmune rabbit antiserum to Mu rIFN-γ was applied; and bound material was eluted with a sodium citrate buffer, pH 3.0. These antisera were kind gifts of Dr. C. Benton, Genentech, Inc. The interspecies rat monoclonal antibody to Mu IFN-γ R4-6A2 was a generous gift of Dr. E. A. Havell, and was supplied as a tissue culture Sn.

T-depleted Spleen Cell Preparations. To prepare B cell–enriched populations, unimmunized mice were inoculated intraperitoneally with 0.05 ml ATS 2 d before harvesting of the spleens. The spleen cell suspension was washed three times and resuspended at one spleen equivalent per 5 ml medium containing anti-Thy-1.2 antibody and rabbit complement (C′) (Hazleton Dutchland, Inc., Denver, PA) diluted 1:15. The cells were incubated for 45 min at 37°C and collected by centrifugation, and the treatment repeated. The cells were then resuspended in 2.5 ml of complete medium, and plastic-adherent cells (two spleen equivalents per dish) were depleted by incubation at 37°C for 2 h in 10-cm-diam petri dishes (Costar, Data Packaging Corp., Cambridge, MA). Nonadherent cells were recovered by gentle flushing of the dishes and resuspended at 1 ml per yield of one petri dish. This volume was applied at room temperature to a Sephadex G-10 column equilibrated with medium (22). Effluent cells were collected by centrifugation, resuspended in complete medium, and counted before use.

Cell Culture. B cell responses were generated in 96-well, flat-bottom microculture plates (Costar) at a density of 10^6 B cells per well, in 0.2 ml of medium with 2×10^6 SRBC. PFC were usually counted after 4 d. Results are expressed as the arithmetic mean ± SEM of triplicate cultures unless otherwise stated (for controls, six replicate cultures were prepared). TRF(E-N) was normally used at a final concentration of 20%. IL-1, from 24 h Sn of silica-stimulated, plastic-adherent human peripheral blood mononuclear cells, was added to all cultures at a final concentration of 5 U/ml, as optimized in preliminary experiments.

RBC Plaque Assay. PFC per culture were determined by following previously described methods (23). Briefly, cultures were sedimented by centrifugation and resuspended in 0.25 ml of complete medium. 0.025 ml of the cell suspension was combined with equal volumes of medium, a 5% SRBC suspension, and absorbed (10% vol SRBC or 20% BRBC for 1 h at 0°C) guinea pig complement, diluted 1:2. Loaded Cunningham slides were incubated at 37°C for 30 min and PFC were counted.

NK Cell Assay. After 4 d of culture, cells were collected and tested for NK activity with in vitro propagated YAC-1 or P-388D_1 cells labeled with ^{51}Cr. Percent specific release was calculated in the standard manner as percent specific lysis = [(experimental cpm − spontaneous release cpm)/(total cpm − spontaneous release cpm)] × 100. Lytic units were determined by interpolation of the dose response curve for the effector/target (E/T) ratio

that produced 30% lysis. The activity of each population is expressed as the number of lytic units per 10^7 effector cells. For example, 10 lytic units is equivalent to 7.5% specific lysis of target cells at an E/T ratio of 25:1.

Results

TRF Activity of an IL-2-rich Lymphokine Fraction. In preliminary experiments we found that a mitogen-free fraction of the Sn of activated human peripheral blood mononuclear cells (PBMC), termed TRF(E-N), acted as a potent TRF for murine spleen cells. The cells were prepared from mice that had received ATS in vivo, and were treated twice with monoclonal anti-Thy-1.2 and C′ and depleted of plastic- and Sephadex G-10–adherent cells. As shown in Fig. 1, TRF(E-N) reconstituted the PFC response to SRBC of splenic B cells from (DBA/2 × CBA/N)F_1 male mice but not that of the reciprocal hybrid. TRF(E-N) showed considerable helper activity at concentrations of 1–5% in the culture and was only moderately dependent on the addition of exogenous IL-1, perhaps because of the cell density of the cultures. In almost all experiments, the above methods produced high PFC responses to SRBC, generally 1,000–1,500 PFC per culture. Similar results were obtained after more stringent T cell depletions, in addition to the ones described. These included in vivo and in vitro treatment with anti-asialo GM_1 serum, and in vitro treatment with monoclonal anti-Lyt-1, -Lyt-2, -Qa-4, and -Qa-5 antibodies in the presence of appropriate enhancing sera and C′, followed by B cell enrichment by positive selection for Ig-bearing cells (not shown). As shown below, PFC responses that were supported by TRF(E-N) were fully antigen specific and were also generated with spleen cells from nu/nu mice (not shown). Thus, human TRF(E-N) exhibits properties concordant with those of the previously characterized murine TRF preparations.

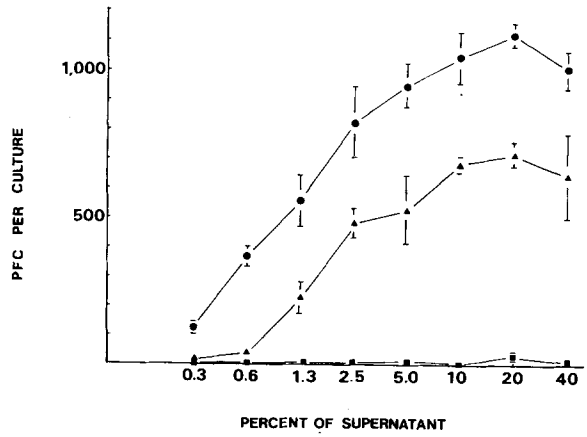

FIGURE 1. Response of normal and immunodeficient CBA/N hybrid mice to human-derived TRF(E-N) and the effect of IL-1. T-depleted spleen cells were prepared from (DBA/2 × CBA/N)F_1 (▲) or (CBA/N × DBA/2)F_1 (■) male mice and cultured for 4 d in the presence of SRBC, 2×10^6 per well, and TRF(E-N). IL-1 at 1.0 U per culture was added to the (DBA/2 × CBA/N)F_1 (●) and (CBA/N × DBA/2)F_1 culture. No PFC were obtained with the latter group in the absence of IL-1. Direct PFC to SRBC were determined at 4 d and expressed as the mean of triplicate cultures ± SEM. Control values, mean (SEM): spleen cells alone, 3 (3); plus SRBC, 0; plus TRF(E-N), 7 (7).

Rabbit Antiserum to Mu IFN-γ Inhibits TRF(E-N) Action. The requirement for IFN-γ as a mediator in these spleen cell responses was tested by adding to the cultures a hyperimmune rabbit antiserum to highly purified Mu rIFN-γ. As shown in Fig. 2, the addition of 5% rabbit antiserum to Mu IFN-γ totally inhibited the production of PFC. A substantial inhibition was still seen at 0.2% (1:500). The IFN-γ-neutralizing activity at this latter dilution was 25 U per microculture (0.20 ml), which was selected for routine use in most subsequent experiments for reasons of economy. Control rabbit serum, obtained from the same animal before immunization, was inactive, and serum from a rabbit immunized to Hu rIFN-γ (8×10^4 neutralizing U/ml) also had no effect.

Inhibition of Murine TRF(E-N) by Specific Ig or Monoclonal Antibody and Reversal With Mu rIFN-γ. Confirmation that antibody to IFN-γ was the active component in the antiserum to IFN-γ was obtained with an affinity-purified antibody eluted from a column of insolubilized Mu rIFN-γ. As shown in Table I, the addition of purified anti-IFN-γ antibody inhibited responses of spleen B cells whereas normal rabbit Ig did not. In this experiment TRF from a murine MLR as well as the human-derived TRF(E-N) were inhibited by antibody to Mu IFN-γ.

It was postulated that abrogation of the PFC response was caused by the quantitative neutralization of natural IFN-γ activity, which was required for PFC responses. Such a mechanism should be reversible upon the addition of excess IFN-γ. This was tested by adding purified rIFN-γ to the cultures (Table II). The

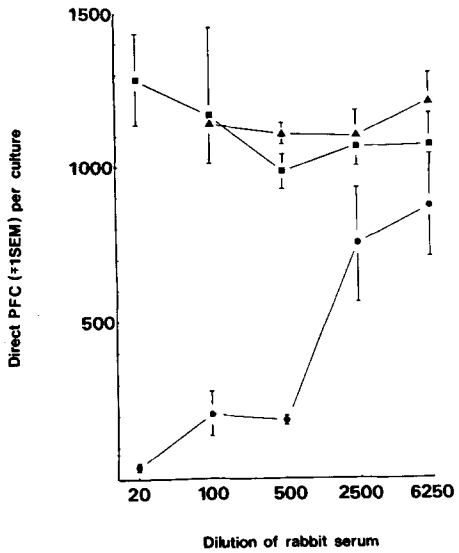

FIGURE 2. Inhibition of TRF-induced PFC by antiserum to Mu IFN-γ. T-depleted spleen cells from (DBA/2 × CBA/N)F_1 mice were cultured for 4 d in the presence 20% TRF(E-N) and SRBC. Rabbit antisera were added at the start of the cultures at the indicated final dilution. The sera are from (■) rabbit MI-3 before immunization, (●) rabbit MI-3 hyperimmunized with Mu rIFN-γ, and (▲) rabbit 12R1 hyperimmunized with Hu rIFN-γ. A 1:20 dilution of MI-3 antiserum is equivalent to 625 IFN-γ viral neutralizing units per culture. Control values: spleen cells alone, 0; plus SRBC, 13 (8); plus TRF(E-N), 16 (4); plus TRF(E-N) and SRBC, 1,363 (211).

TABLE I

Antibody to IFN-γ Inhibits the TRF Activity of Murine MLR Sn and TRF(E-N)

Antigen	Purified antibody to Mu IFN-γ (ng/well)	PFC per culture (SEM)	
		TRF (MLR)	TRF(E-N)
SRBC	0	1,087 (92)	1,038 (42)
SRBC	250	57 (17)	90 (12)
SRBC	83	423 (84)	667 (58)
SRBC	28	613 (52)	893 (132)
SRBC	9	680 (81)	937 (109)
	Normal Ig (ng/well)		
Controls:			
SRBC	1,000	1,060 (49)	907 (66)
—	—	10 (6)	55 (10)

Murine T-depleted spleen cells were prepared as described in Materials and Methods except plastic adherence and Sephadex passage was omitted. 10^6 cells were cultured for 4 d in the presence of 25% murine or 20% human TRF(E-N) Sn. Immunospecifically purified antibody to Mu rIFN-γ was added at the start of the cultures. MLR Sn was the supernatant of a 24 h murine MLR. Antibody was present from the start of the cultures and had 10 U of antiviral neutralizing activity per 100 ng.

PFC responses that were supported by either murine or human TRF were strongly inhibited in a dose-dependent fashion by antiserum or purified antibody to IFN-γ. These inhibited responses were substantially or fully reconstituted by the addition of 2,500 U of Mu rIFN-γ.

The stoichiometry of the anti-IFN-γ effect was examined in greater detail in a dilution analysis of both the antiserum to Mu IFN-γ and the rIFN-γ that was added to the cultures. A comparison of the inhibitions caused by 25 and 125 U of antiserum (Table III) indicates that, with lower doses of antibody, recovery is more readily obtained when a similar amount of IFN-γ is used. The addition of IFN-γ to TRF-containing cultures in separate experiments had no effect on TRF-mediated responses. Suprisingly, IFN-γ also failed to augment weak PFC responses induced by suboptimal concentrations of TRF, suggesting that some other cytokine was limiting in the system (Tables IV and V).

It is also important (Table III and Fig. 2) that antiserum to human IFN-γ has little or no inhibitory activity, and that human rIFN-γ cannot reconstitute the inhibited response, although it significantly augments the PFC response of noninhibited cultures. Thus the activity of human-derived TRF(E-N) cannot be explained by its content of Hu IFN-γ. The Sn of the Gibbon lymphoma MLA-144, which constitutively produces IL-2, exhibited high TRF activity in these cultures (Table V). Addition of antibody to Mu IFN-γ inhibited those PFC responses. The responses were restored by the addition of 2,500 U of rIFN-γ. No direct TRF effects of rIFN-γ alone were detected in these studies. Very recently (17) a monoclonal antibody to Mu IFN-γ was generated that can functionally neutralize the biological properties of IFN-γ. When added to the

TABLE II

Antiserum or Specific Antibody to IFN-γ Inhibits the Action of Human TRF(E-N) or Murine (Con A) TRF on B Cell Responses to SRBC and Reversal of the Effect with Mu rIFN-γ

Experiment 1 TRF	Antigen	Antiserum to murine IFN-γ (units)	Addition of Mu rIFN-γ (2,500 U)	PFC to SRBC per culture (SEM) of 10^6 B cells
Con A/Sn	SRBC	0	−	1,020 (70)
Con A/Sn	SRBC	50	−	157 (35)
Con A/Sn	SRBC	6.3	−	203 (77)
Con A/Sn	SRBC	3.1	−	313 (72)
Con A/Sn	SRBC	1.6	−	290 (100)
Con A/Sn	SRBC	0.8	−	583 (198)
Con A/Sn	SRBC	0	−	920 (52)
Con A/Sn	SRBC	25	−	210 (55)
Con A/Sn	SRBC	25	+	730 (107)
Con A/Sn	No SRBC	0	−	25 (9)
TRF(E-N)	SRBC	0	−	967 (111)
TRF(E-N)	SRBC	25	−	423 (50)
TRF(E-N)	SRBC	25	+	935 (72)
TRF(E-N)	No SRBC	0	−	12 (6)
None	SRBC	0	−	7 (2)

Experiment 2 TRF	Antigen	Purified antibody to Mu IFN-γ (ng)	Addition of Mu rIFN-γ (2,500 U)	PFC to SRBC per culture (SEM) of 10^6 B cells
TRF(E-N)	SRBC	0	−	1,290 (64)
TRF(E-N)	SRBC	1,000	−	87 (33)
TRF(E-N)	SRBC	500	−	267 (101)
TRF(E-N)	SRBC	250	−	557 (142)
TRF(E-N)	SRBC	125	−	883 (151)
TRF(E-N)	SRBC	63	−	1,013 (219)
TRF(E-N)	SRBC	500	+	1,243 (87)
		Normal rabbit Ig (ng)		
TRF(E-N)	SRBC	1,000	−	1,350 (53)
None	SRBC	0	−	0
None	None	0	−	0
TRF(E-N)	None	0	−	23 (12)

T-depleted spleen cells were cultured at 10^6 cells per microculture for 4 d in the presence of 25% murine Con A Sn or 20% human TRF(E-N) Sn. Mu rIFN-γ, (Exps. 1 and 2), rabbit antiserum (Exp. 1), or immunospecifically purified Ig (Exp. 2) were added at the indicated concentration per well, at the initiation of the cultures. 1,000 ng of antibody had 100 antiviral neutralizing units of activity.

TABLE III

Dose Response Analysis of Inhibition of TRF Activity by Antibody to Mu IFN-γ and Recovery of Response by Addition of rIFN-γ

Source of rIFN-γ	Units of rIFN-γ added	No antibody	Direct PFC to SRBC per culture (SEM)		
			TRF(E-N) plus antibody to Mu IFN-γ		TRF(E-N) plus antibody to Hu IFN-γ
			25 U	125 U	125 U
Murine	0	0	457 (135)	247 (28)	1,083 (50)
	2.5	0	337 (58)	307 (93)	1,053 (107)
	25	17 (17)	430 (50)	360 (106)	1,787 (527)
	250	13 (12)	860 (111)	293 (47)	1,640 (280)
	2500	13 (9)	1,263 (142)	1,000 (27)	1,657 (303)
Human	0	0	497 (154)	327 (55)	1,277 (274)
	2.5	0	377 (88)	297 (29)	1,693 (124)
	25	6 (6)	353 (160)	247 (43)	1,467 (162)
	250	3 (3)	280 (26)	220 (30)	2,027 (135)
	2500	17 (17)	387 (33)	257 (29)	2,320 (183)
Controls: SRBC plus TRF(E-N)			1,474 (80)		
SRBC only			0		

T-depleted spleen cells were cultured for 4 d in the presence of SRBC and TRF(E-N). Antibody and/or rIFN-γ were added at the start of the cultures. Amounts of antibody and IFN-γ added are expressed in units per 0.2 ml culture.

TABLE IV

rIFN-γ Lacks TRF Activity and Does Not Complement Limiting Concentrations of TRF(E-N) for PFC Responses

Units of Mu rIFN-γ	PFC (SEM) per culture				
	Percent TRF(E-N) per culture:				
	0	2.5	5	10	20
2,500	20 (12)	770 (106)	867 (96)	1,437 (108)	1,400 (202)
250	7 (7)	853 (87)	893 (107)	1,533 (81)	1,187 (78)
25	10 (6)	817 (64)	1,073 (47)	1,300 (126)	1,487 (94)
2.5	20 (10)	913 (62)	1,073 (108)	1,163 (231)	1,373 (54)
0	90 (25)	967 (52)	1,260 (31)	1,327 (153)	1,450 (130)

10^6 T-depleted spleen cells were placed in culture with 2×10^6 SRBC. TRF(E-N) and Mu rIFN-γ were added to the cultures as indicated. PFC were determined after 4 d. Results are expressed as mean PFC (SEM) per culture. Control values: None, 3 (2); plus TRF(E-N), 17 (5).

TRF assay, this antibody showed potent inhibitory effects (Table VI) which were reversed by the addition of excess Mu rIFN-γ.

Specificity of the PFC Responses After Reversal of Antibody Block by rIFN-γ. The PFC responses supported by TRF(E-N) were shown in preliminary studies to be antigen specific. However, it was possible that the PFC responses produced by the reversal of anti-IFN-γ antibody by excess rIFN-γ were not antigen specific, and could have been due to polyclonal B cell activation by rIFN-γ. The results

TABLE V
Constitutive Production of TRF by a Primate Lymphoma MLA-144

Percent of MLA-144 Sn	PFC per culture (SEM)			
	Plus SRBC	Plus rIFN-γ and SRBC	Plus anti-IFN-γ and SRBC	Plus anti-IFN-γ, rIFN-γ, and SRBC
25	1,043 (43)	943 (203)	310 (70)	1,060 (128)
12.5	840 (108)	623 (60)	143 (47)	537 (223)
6.25	230 (60)	177 (102)	40 (10)	350 (125)
Controls				
None	2 (2)	—	—	—
TRF(E-N)	1,267 (60)	—	—	—

10^6 T-depleted spleen cells were cultured for 4 d in the presence of MLA-144 Sn. rIFN-γ (2,500 U) and/or RAM-IFN-γ (25 neutralizing units) was added to indicated cultures. Direct PFC were determined and the results expressed as the mean (SEM) of triplicate cultures.

TABLE VI
Inhibition of TRF Activity by Monoclonal Antibody to Mu IFN-γ and Recovery of the Response With rIFN-γ

Source of rIFN-γ (2,500 U)	Culture Sn dilution	Direct PFC (SEM) to SRBC per culture	
		Monoclonal R4-6A2 Sn to Mu IFN-γ	Culture Sn of spent NS-1 cells
None	5×	87 (29)	1,163 (159)
None	1×	417 (78)	1,187 (73)
Murine	5×	1,140 (241)	ND*
Murine	1×	1,290 (145)	ND
Controls: SRBC plus TRF(E/N)		1,310 (105)	
SRBC only		2 (2)	

T-depleted spleen cells were cultured for 4 d in the presence of SRBC and 20% TRF(E-N). Sn from the monoclonal line R4-6A2 or from the myeloma P3/NS-1/1-Ag4-1 were used neat or were concentrated 5× using a Centricon 10 membrane (Amicon Corp., Danvers, MA). At the start of incubation some cultures also received Mu rIFN-γ.
* Not done.

of an experiment using SRBC or BRBC antigens (Table VII) show that both the uninhibited and the recovered responses are fully antigen specific.

TRF Induces IFN-γ Production and Potent NK Activity in T-depleted Spleen Cell Cultures. The preceding experiments suggest that IFN-γ is produced in cultures of T-depleted murine spleen cells. This was directly tested using Sn from spleen cells cultured in various conditions (Table VIII). The data demonstrate that IFN-γ is produced only in cultures stimulated with TRF(E-N) and can be detected on day 3, although levels below the sensitivity of the antiviral assay may be present at earlier times. Antiserum to IFN-γ, but not to IFN-α and -β, abolished both the IFN antiviral activity and the PFC response.

Since B cell populations are not thought to produce IFN-γ, we examined the

TABLE VII

Human TRF(E-N) Supports Antigen-specific PFC Responses: Reversal by rIFN-γ of Antibody-induced Inhibition Is Also Antigen-specific

Antigen in culture	TRF in culture	Antibody and/or IFN-γ added	PFC per culture (SEM) measured on:	
			SRBC	BRBC
None	TRF(E-N)	None	72 (26)	0
SRBC	TRF(E-N)	None	1,000 (79)	0
BRBC	TRF(E-N)	None	73 (18)	618 (48)
SRBC	TRF(E-N)	Anti-IFN-γ	300 (36)	8 (8)
BRBC	TRF(E-N)	Anti-IFN-γ	43 (15)	48 (10)
SRBC	TRF(E-N)	Anti-IFN-γ plus Mu rIFN-γ	953 (133)	0
BRBC	TRF(E-N)	Anti-IFN-γ plus Mu rIFN-γ	10 (6)	405 (65)
SRBC	TRF(E-N)	Mu rIFN-γ	878 (99)	2 (2)
BRBC	TRF(E-N)	Mu rIFN-γ	53 (11)	510 (58)
None	None	None	48 (10)	2 (2)
SRBC	None	None	13 (6)	0
BRBC	None	None	20 (7)	0

T-depleted spleen cells were cultured with 2×10^6 SRBC or BRBC for 4 d with additions to the cultures as indicated. 25 U of rabbit antibody or 2,500 U of rIFN-γ were used.

TABLE VIII

TRF(E-N) Induces IFN-γ in T-depleted Spleen Cell Cultures

Culture conditions	Units* of IFN-γ				Mean (SEM) PFC per culture
	Day of assay				
	1	2	3	4	
Medium	0	0	0	0	0
SRBC	0	0	0	0	0
TRF(E-N)	0	0	0	160	0
TRF(E-N) + RAM-IFN-γ	0	0	0	0	3 (3)
TRF(E-N) + SRBC	0	0	160	160	717 (88)
TRF(E-N) + SRBC + RAM-IFN-γ	0	0	0	0	17 (3)

Control: TRF(E-N) in the absence of cells, 0 U IFN

10^6 T-depleted spleen cells were cultured for variable periods of time in the presence of TRF, antigen, and antibody to IFN-γ, as indicated. Supernatants were pooled from triplicate wells and assayed for IFN-γ levels. On day 4, cells from individual wells were assayed for PFC responses. RAM-IFN-γ was added to indicated cultures at 25 neutralizing units per culture. IFN activity was measured by inhibition of vesicular stomatitis virus cytopathic effects on L_B cell monolayers using serial twofold dilutions of supernatant. IFN-γ was identified in each of the active supernatants by sensitivity to pH 2.0, and neutralization with monoclonal R4-6A2 antibody and RAM-IFN-γ serum, but not with RAH-IFN-γ antibody or antibody to Mu IFN-α, -β. The assays were standardized by inclusion of a control Mu IFN-α, -β preparation (NIH reference reagent No. G002-904-511).

* 1 U of IFN as determined in this assay is equivalent to 0.08 IU/ml.

extent of non-B cell contamination in the spleen cell cultures. At the start of culture, <1% (generally 0–0.6%) Thy-1.2$^+$ cells were routinely detected, but, by day 4, 5–10% of the cells were Thy-1.2$^+$. Since NK cells and their precursors (*a*) show variable expression of Thy-1 (24), (*b*) proliferate and mature in cultures of T-depleted spleen cells in the presence of IL-2 (24), and (*c*) produce abundant IFN-γ on stimulation with human (25) or murine (26) IL-2, we examined the PFC cultures after 4 d for NK cell activity. As shown in Table IX, a dramatic appearance of NK cells is evident. This effect requires stimulation of the cultures with TRF. Lysis of YAC-1, but not P-388D$_1$, target cells confirmed the NK character of the effector cells. Thus, TRF(E-N) induces both IFN-γ production and the striking NK cell activity of T-depleted murine spleen cells.

Time of Action of IFN-γ in the TRF Assay. To investigate the time at which human TRF(E-N) and IFN-γ act in this system, we measured PFC responses after adding TRF(E-N) at different times, adding antibody at different times, and varying the time of reversal of inhibition with Mu rIFN-γ. As shown in Table X, TRF(E-N) was most active when included at the beginning of culture or at day 1. Antibody to Mu IFN-γ inhibited responses only if added very early. When antibody to Mu IFN-γ was added on day 1, only a partial inhibition was

TABLE IX

TRF(E-N) Induces the Maturation of Potent NK Activity

Exp. No.	Target cell	Lytic units before culture	Lytic units after culture with:			
			None	SRBC	TRF(E-N)	SRBC plus TRF(E-N)
1	YAC-1	2	0.3	0.6	667	1,000
2	YAC-1	4	7	5	196	147
	P-388D$_1$	ND	0	4	11	4

NK cell activity was examined in assay cultures using 10^4 YAC-1 or P-388D$_1$ target cells labeled with ^{51}Cr. Results are expressed in lytic units per 10^7 effector cells, as described in Materials and Methods.

TABLE X

Kinetic Analysis of TRF Action and Inhibition With Antiserum to Mu IFN-γ

Addition to cultures		PFC (SEM) to SRBC per culture with variable component added on days:			
Continuously present	Variable component	0	1	2	3
Nil	Nil	0			
Nil	rIFN-γ	6 (3)	0	0	3 (3)
Nil	TRF(E-N)	2,157 (18)	2,153 (63)	423 (107)	3 (3)
TRF(E-N)	Nil	1,917 (237)			
TRF(E-N)	RAM-IFN-γ	273 (48)	1,153 (13)	2,017 (108)	2,077 (47)
TRF (E-N) plus RAM-IFN-γ	Nil	427 (52)			
TRF(E-N) plus RAM-IFN-γ	2,500 U rIFN-γ	2,017 (110)	1,913 (185)	1,170 (119)	377 (26)

T-depleted (DBA/2 × CBA/N)F$_1$ spleen cells were cultured with SRBC for 4 d. At the start of the culture or at various times thereafter the indicated additions were made.

obtained. Finally, in cultures where responses were inhibited by antibody to IFN-γ, the responses recovered only when Mu rIFN-γ was added during the first 2 d of culture. This last result shows that the specific IFN-γ component of the signal for TRF activity must be received early in the response, and that it probably was responsible for the kinetics in the first two groups of results.

The inhibition of TRF-supported responses by antibody to IFN-γ is apparently caused by a delay in the kinetics of the PFC response (Table XI). This result is expected if endogenous IFN-γ production by spleen cells can saturate the capacity of the inhibitory antibody and if a critical period for IFN-γ action has not lapsed.

Discussion

In this report we have examined the role of IFN-γ in TRF-dependent PFC responses of murine spleen cells to red blood cell antigens. Several antibody preparations with high specific neutralizing activity for human and murine IFN-γ were added to the cultures to test their effects on TRF action. The results show that antibody to murine but not to human IFN-γ can abrogate the TRF activity of either human, primate, or murine TRF preparations and that these inhibitions are reversed specifically by the addition of excess murine but not human recombinant-derived IFN-γ.

Although TRF activity was described more than a decade ago (27), until recently there had been little progress in the molecular identification of the active factor(s), probably because, as shown by new evidence, several distinct factors cooperate to mediate TRF effects. In addition to SRBC responses, TRF is active in the antibody responses of B cells to hapten-protein antigens (12) and thymus-independent antigens (13, 28) and in polyclonal activation of B cells by anti-Ig antibodies (4). Thus, TRF appears to be the major cytokine-controlled regulatory pathway for the expansion and maturation to antibody-producing cells of a major, Lyb-5^+ subpopulation of B cells (29). Studies (3, 8–10) using new methods for lymphokine purification and Sn of long-term or cloned T cell lines in factor reconstitution experiments have produced clear evidence for the presence of several required components in TRF. Collectively, the data indicate that, for the systems studied, at least two major components are involved: first,

TABLE XI

Antibody to IFN-γ Delays the Generation of PFC

Source of TRF	Antibody to IFN-γ	PFC per culture (SEM)			
		Day of assay			
		3	4	5	6
TRF(E-N)	−	10 (6)	767 (171)	1,330 (91)	1,123 (105)
TRF(E-N)	+	0	137 (72)	1,160 (57)	1,240 (77)
Con A Sn	−	0	977 (221)	1,090 (58)	55 (25)
Con A Sn	+	0	153 (62)	750 (91)	990 (228)
No TRF	−	0	0	7 (3)	7 (7)

10^6 T-depleted spleen cells were cultured in the presence of the indicated source of TRF and 2×10^6 SRBC. 25 neutralizing units of RAM-IFN-γ were added to some cultures. Specific PFC were determined on the day indicated. Results are expressed as the mean PFC (SEM) per culture.

an IL-2-containing Sn of T cells, which may, however, include additional mediators, and second, an IL-2-depleted Sn of Con A–activated spleen cells (8), which may also be replaced by secreted products of some T cell lines (10, 11, 30). In some studies, IL-2-containing Sn alone are active as TRF, possibly due to the activity of this lymphokine on contaminating T cells or other non-B cells (2); generally, however, TRF activity of IL-2 alone is not reported in other systems. Recent work has shown that the second component has an apparent molecular weight of 40,000 (8) and that its activity is most evident when added 24 h after the start of cultures (11, 12), as noted previously (31) for unfractionated Sn of Con A–stimulated spleen cells. This second component was shown (1, 12, 14) to be sensitive to treatment at pH 2 and to correlate with IFN-γ activity in Sn of T cell lines. In a recent report (15) rIFN-γ showed similar biological activity. IFN-γ is already known to act as a differentiation signal in several murine immunological systems, such as the induction of major histocompatibility complex (MHC) antigens (32), the activation of mononuclear phagocytes (17, 18, 33), the enhancement of NK cell activity (25), and a TRF-like action in the genesis of cytotoxic T cells (34). However, the regulation of B cell differentiation represents a new and important biological function of IFN-γ.

To examine the role of IFN-γ in antibody responses supported by TRF, we used the same preparations of antibody to IFN-γ shown to functionally neutralize the antiviral and macrophage-activating factor (MAF) activity of IFN-γ (17, 18). Other antibodies to IFN-γ have been shown to abrogate TRF help for cytotoxic T cells (35) and, recently, the induction of MHC class II antigens by IFN-γ was shown to be inhibited by antibody to IFN-γ (19). Experiments using antibody-mediated inhibition are able to demonstrate a necessary role for a specific lymphokine in a mixture of biologically active molecules in a crude Sn. In the present study, this method was able to determine whether IFN-γ is a necessary component of TRF action, in a variety of TRF preparations. Although previous reconstitution studies, using monoclonal cytokines or recombinant-derived proteins, support a role for IFN-γ, it is not clear that IFN-γ is normally a required component of TRF. It is possible that IFN-γ substitutes for, or bypasses the need for, other mediators present in TRF that normally support antibody responses. The results in the present study consistently show that strong, specific inhibitions are obtained using four independent sources of TRF, demonstrating that IFN-γ is consistently required for the activity of TRF.

A human, IL-2-rich lymphokine fraction, known to have TRF activity on human B cells (36), is herein shown to be an effective TRF for murine B cells and to resemble murine TRF, in magnitude, kinetics, and specificity, in its support of murine PFC responses to SRBC. Moreover, this preparation was inactive with B cells from mice with the *xid* defect, as shown for murine TRF (6), suggesting that common TRF pathways are followed. In view of the need for IFN-γ in the action of murine TRF, as well as the species specificity of the biological actions of IFN-γ (18), it was surprising that human-derived TRF was active in this system. However, human IFN-γ appears to have no role in these responses, since rabbit antibody to Hu IFN-γ and four monoclonal antibodies with high Hu IFN-γ neutralizing activity (data not shown) did not suppress TRF activity, whereas antibody to Mu IFN-γ did inhibit PFC production. The specific

lymphokine target of this latter antibody was examined in several ways. First, we found that antiserum to Mu IFN-γ had no effect on human IL-2 and B cell growth factor (BCGF) in their respective functional assays (data not shown). Second, the inhibited responses were restored by the addition of excess murine but not human rIFN-γ. Third, an antigen affinity-purified rabbit Ig, specific for Mu rIFN-γ, was similarly inhibitory. This result indicates that the inhibitions could be due solely to antibodies to Mu rIFN-γ rather than to other antibodies in the sera. Fourth, a monoclonal antibody that specifically neutralizes the antiviral effect of Mu IFN-γ also abrogated TRF activity, and this block was relieved with excess Mu IFN-γ. This result argues strongly that Mu IFN-γ is the target lymphokine of the antibodies, since it is less likely that a monoclonal antibody would avidly crossreact with another lymphokine (a possibility to be considered with polyvalent antisera). The finding that Hu rIFN-γ did not restore the response corresponds both with the specificity of the antisera and with the species specificity of its biological effects, e.g., it has no antiviral activity when tested in murine assays (18). Moreover, in other experiments, the functional activity of TRF(E-N) was unaltered after exposure to pH 2.0.

The reversal of the inhibition of PFC responses upon addition of rIFN-γ suggests that quantitative relationships in this system may be complex. It appears that >250 U of IFN-γ are required to reverse the inhibition produced by 25 neutralizing units of antibody. This discrepancy may occur because IFN-γ added at the initiation of the cultures may be substantially degraded or absorbed in the high density cell cultures before its time of action, which may extend to 48 h. Time-dependent absorption of IFN-γ in murine cell cultures has been demonstrated (37).

Together, our results argue that IFN-γ is necessary in TRF action, even in the case of a TRF that genetically lacks this factor; in the latter circumstance, IFN-γ is produced by the cultured, T-depleted murine spleen cells. Recent work on the production of IFN-γ by spleen cells has shown that unfractionated spleen cells (38) as well as nu/nu or conventional T-depleted spleen cells (25) produce IFN-γ upon culture in the presence of purified murine or human IL-2. NK cells appear to be a very adequate source of IFN-γ (26). In the present study (Table VIII), significant levels of Mu IFN-γ were recovered from the culture Sn after 3–4 d. Since some biological systems, e.g., MAF assays, are considerably more sensitive to IFN-γ than antiviral assays (18), it is possible that IFN-γ concentrations adequate to support B cell responses are present at earlier times. This possiblity is favored by the high local concentrations of IFN-γ that occur in cell-to-cell interactions. It also appears that IFN-γ is not produced constitutively but is induced by TRF. The cellular source of this factor remains unclear but may be the T-related, large granular lymphocytes that possess NK activity and are reduced but usually not fully eliminated in most T cell depletions. These cells may also comprise the component that permits TRF activity of FS6-14.13 Sn (2) or may be the novel helper cells postulated by Parker (10) to explain a possible mode of action of IL-2 in TRF-supported PFC responses in the anti-Ig system. Residual T cell activity in T-depleted spleen cell populations has been well documented (10, 39). In the present study, high levels of NK cells were produced (Table IX) after 4 d of culture in the presence of TRF(E-N). In view of the

ability of these cells to produce IFN-γ (25, 26), they may play a crucial role in TRF-mediated B cell responses. We found that antibody to IFN-γ did not permanently inhibit TRF-supported anti-SRBC responses but rather delayed the appearance of PFC (Table XI). The similar magnitude of the intact and delayed responses suggests that the antibody acts as a transient sink for IFN-γ produced in the cultures, and that the antibody capacity is soon surpassed by endogenous IFN-γ production.

In other experiments (Table V), an IL-2-rich Sn constitutively produced by the Gibbon T cell line MLA-144 (supplied by Dr. H. Rabin), when used at a similar dilution of IL-2 activity, was similar to TRF(E-N) for TRF activity. This result suggests that any potentially copurified but undetectable mitogens are not responsible for the biological effects of TRF(E-N). Interestingly, Mu rIFN-γ showed no TRF activity in these experiments, either alone (Tables III and X) or in the presence of suboptimal concentrations of TRF(E-N) or TRF(MLA-144) (Tables IV and V). This suggests that IFN-γ is not limiting in the latter cultures or is functionally inactive but antigenically intact. The latter seems unlikely since the molecule was functional in several independent biological assays. The lack of TRF activity of IFN-γ alone is consistent with the need for at least two factors for TRF activity, the second of which may be supplied by the IL-2-rich TRF(E-N).

Our data do not address the role of IL-2 itself in these TRF-dependent responses. IL-2 may act in synergy with IFN-γ, or it may, secondarily, induce the production of IFN-γ (25, 26) and perhaps other factors such as BCGF (30, 40). Alternatively, it is possible that other factors, such as BCGF, that copurify with human IL-2 (41) are directly involved in these responses. Human IL-2 is known (10) to be active on murine T cells and has been shown to replace murine IL-2 in both cell expansion and antibody production by T-depleted murine spleen cells (10). While most past studies have used IL-2-containing Sn of T cell lines, which may contain other factors such as BCGF (1, 10, 42, 43) that may be needed for maximal responses, the affinity-purified human IL-2 used by Parker (10) argues most strongly for a role of IL-2 in the TRF system. This role may be direct and necessary, such as a signal for B cell differentiation, or may be indirect, such as inducing other factors that are the ultimate mediators of signals needed for B cells. The production of secondary mediators induced by IL-2, or by TRF, as in the present study, may account for some of the differing results concerning the controversial role for IL-2 in TRF action. Such effects could be variable and may depend significantly on the purity of the cells and the factors used (3, 28). A recently described (19) intermediate modulator for Ia antigen expression induced by IFN-γ is an example of this concept. Some of these uncertainties could be resolved in studies with monoclonal antibodies that neutralize the activity of specific lymphokines such as BCGF, IL-1, and IL-2. Alternatively, antibodies to the cellular receptors for these lymphokines may serve the same purpose, and help to identify the cell subsets involved. Single-cell assays may be the most useful way to analyze the distinct action of lymphokines on B cells. Such cultures would be free of the products of all forms of accessory cells.

Summary

The role of gamma interferon (IFN-γ) in T cell–replacing factor (TRF) activity for antigen-specific plaque-forming cell (PFC) responses in vitro was studied using antibodies to murine IFN-γ (Mu IFN-γ). TRF activity was present in supernatants (Sn) of Con A– or mixed leukocyte reaction–stimulated murine spleen cells as well as in an IL-2-rich fraction of phytohemagglutinin-stimulated human peripheral blood lymphocyte Sn and in the Sn of the Gibbon T lymphoma MLA-144. The human TRF was highly active with cells from nu/nu mice and normal mice but not with cells from animals with the *xid* immunologic defect, similar to the activity of murine TRF. Antibodies to IFN-γ consisted of hyperimmune rabbit antisera, IFN-γ affinity-purified rabbit immunoglobulin and an interspecies hybridoma specific for Mu IFN-γ. The results show that the activities of all preparations of TRF are markedly diminished or abrogated by antibody to Mu IFN-γ but not by antibodies to human IFN-γ (Hu IFN-γ), nor by normal rabbit sera or purified rabbit Ig. The degree of inhibition was dose dependent and was quantitatively reversed by the addition to the cultures of recombinant-derived Mu IFN-γ (Mu rIFN-γ) but not Hu rIFN-γ. This reversal was fully antigen specific and thus not attributable to polyclonal B cell activation by IFN-γ, which is inactive alone in the TRF assay. Kinetic analysis shows that IFN-γ must act by 24–48 h to produce PFC responses at 4 d. Together, the data demonstrate that IFN-γ is a necessary mediator for TRF effects and that IFN-γ is induced by TRF from T-depleted murine spleen cells in sufficient quantity to support large antibody responses. The source of this IFN-γ may be the potent natural killer cells that are induced in cultures stimulated with TRF.

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