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CELLULAR REGULATION OF IMMUNE INTERFERON PRODUCTION

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Spleen cells obtained from mice injected 24–72 h previously with a T-cell mitogen were poor producers of immune interferon (IFNγ) when restimulated with the same mitogen in vitro. The reduced response appeared to be due to a suppressor cell population, since virgin spleen cells also gave a reduced response when cultured with the cells from injected mice. Through the use of different concentrations of monoclonal anti-Thy-1 antibody, the mitogen-induced suppressor cell population was shown to contain a relatively high density of the Thy-1 antigen. The IFN-producing cell population contained a relatively low density of the Thy-1 antigen, and either evolved from cells with a high density of Thy-1 antigen or required such cells as helpers in production of IFNγ.

A model is proposed according to which the induction of T cells in mice for IFNγ production then involves a cell population with a relatively high density of Thy-1 antigen. The induced cells appear to evolve into IFN-producing cells of low density of Thy-1 antigen. IFN production by the cells with low density of Thy-1 antigen is suppressed or regulated by another stimulated cell population that, like the virgin T cells, possesses a high density of Thy-1 antigen. The IFN-producing cells with low density of Thy-1 antigen appear to represent a small portion of the total T-cell population.

Thy-1 lymphocytes feedback regulation lymphokine suppressor cells

INTRODUCTION

The effects of interferon (IFN) on cell function may be expressed in the form of antiviral, anticellular, immunoregulatory, and antitumor activities. Three antigenic types of IFN have been identified in the human and mouse systems, and have been designated as IFNα, IFNβ, and IFNγ [15]. IFNα and IFNβ are classically induced in cells by viruses or synthetic polyribonucleotides [6, 9]. IFNγ is induced in antigen primed lymphocytes upon subsequent exposure to the specific antigen [8, 14] and in unprimed lymphocytes with T-cell mitogens [4, 11, 16, 18]. IFNs produced in lymphocytes are, by definition, lymphokines.

Little information is available on the events that regulate the induction and action of lymphokines. Therefore, we decided to examine the cellular events associated with the regulation of the induction and/or action of the lymphokine IFNγ. We have determined that a regulatory cell is activated during the IFNγ response. The density or accessibility of the Thy-1 antigen on this regulatory cell differs from that of the cell actually produc-

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ing the IFNγ. The findings are significant in that IFNγ is felt to play an important role in viral infections, in the regulation of cell proliferation and of the immune response [2, 10, 12].

MATERIALS AND METHODS

Mice

C57B1/6 female mice, 8–12 weeks old, were obtained from Jackson Laboratories, Bar Harbor, Maine.

Staphylococcal enterotoxin A (SEA)

Purified SEA was employed as the inducer of IFNγ. It was produced by the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, Ohio. The purity of this SEA preparation is documented elsewhere [1].

In vivo studies

One group of mice was injected intravenously with SEA. At various times, animals were sacrificed by bleeding from the brachial blood vessels. The serum was collected and tested for IFNγ activity. Another group of mice was also injected intravenously with SEA, and at various times the spleens were removed and tested in vitro for IFN production and for their effect on IFN induction in virgin spleen cells.

Treatment of spleen cells with monoclonal antibody to mouse Thy-1.2 antigen

Spleen cells (1 ml at $3–5 \times 10^7$ cells/ml), anti-Thy-1.2 antibody (1 ml of a 10^{-3} or 10^{-4} dilution), and guinea pig complement (0.167 ml of serum) were mixed and incubated at 37°C for 1 h. The cells were washed two times and added to various cultures at a final concentration of 1.5×10^7/ml. Killing of cells as a result of the treatment was 30–40%. The monoclonal anti-Thy-1.2 antibody was obtained from New England Nuclear.

IFN assay

IFN was assayed by a microplaque reduction method, as described, using approximately 40 plaque-forming units (p.f.u.) of vesicular stomatitis virus per well in mouse L cells [11]. In our studies, a concentration of 1 unit/ml of IFNγ is defined as the concentration required to decrease the number of p.f.u./well by 50%. One unit of our IFNγ inhibits virus replication to the same extent as one unit of NIH reference mouse fibroblast IFN. The IFN activity described in this report is IFNγ as determined by neutralization reactions with specific antisera [13].

RESULTS

In vivo induction of IFNγ

A first step in studying the regulatory events was to determine the kinetics of IFN production in mice injected intravenously with SEA. Data from a representative experiment are presented in Table 1. The IFN response peaked in 6–24 h and declined thereafter. The response was generally lower than that obtained by injecting viruses or antigens into preimmunized animals [14]. The purpose here, however, was not to obtain high levels of IFN, but to determine the cellular aspects of regulation of IFNγ production. The data warranted an examination of the spleen cells of injected mice for altered ability to produce IFN.

Kinetics of IFNγ production by spleen cells from mice injected with SEA

Spleen cells obtained from mice 2 h after SEA injection and cultured without further stimulation produced modest levels (about 100 units/ml) of IFNγ in a 'steady-state' manner over a period of 3 days (Fig. 1). When these cells were also stimulated with SEA in vitro, higher yields of IFN were obtained with kinetics similar to the response of spleen cells obtained from uninjected mice (Fig. 1). Cultured spleen cells obtained from mice 24 h after SEA injection did not produce detectable levels of IFN over the 3 day incubation period. When these cells were stimulated in vitro with SEA, the maximum yield of IFN was seen on day 1 with a dramatic reduction by day 3 (Fig. 1). It thus appears that spleen cells obtained from mice 24 h after SEA injection were relatively refractory to sustained production of IFN after further stimulation with SEA in vitro, which was most evident on day 3 in culture.

TABLE 1

Kinetics of production of IFNγ in C57B1/6 mice injected intravenously with SEA[a]

Time (h post injection)	Serum interferon (units/ml)
2	0, 0
4	0, 0
6	44, 34
24	57, 51
48	36, 1
72	11, 1

[a] Mice were injected intravenously with 5 μg SEA. Two mice were injected for each time point and results are presented for the individual mice.

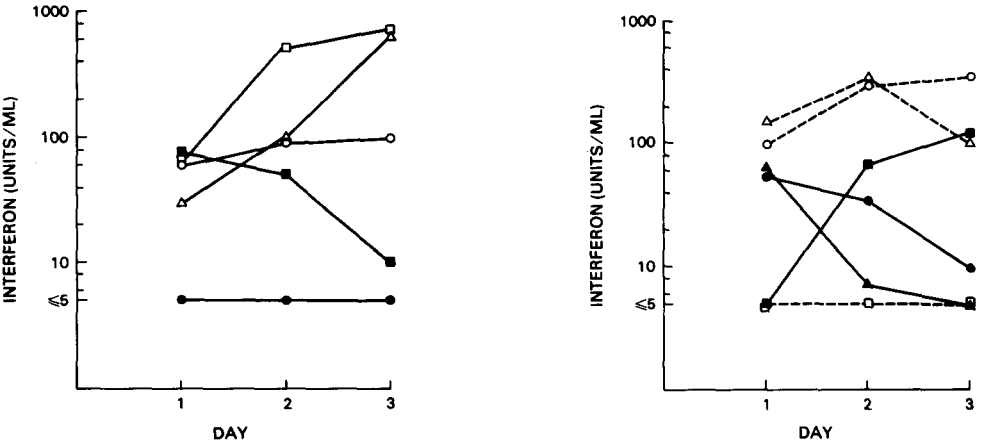

Fig. 1. Kinetics of IFNγ production by cultured spleen cells obtained from mice injected with SEA. Mice were injected intravenously with 5 μg SEA. Spleens were removed at 2 and 24 h after injection and cultured at 1.5 × 10^7 cells/ml in the absence or presence of 0.5 μg SEA/ml. Pooled spleens from 3 mice were used for each time interval. Spleen cell cultures: △, uninjected, SEA added in vitro; ○, 2 h after SEA injection; □, 2 h after SEA injection, SEA added in vitro; ●, 24 h after SEA injection; ■, 24 h after SEA injection, SEA added in vitro. Assays were carried out in duplicate and coefficients of variation were less than 20%.

Fig. 2. Regulation of IFNγ production by spleen cells from mice injected with SEA. Spleen cells obtained from mice injected 24 h earlier with 5 μg SEA were mixed with spleen cells from uninjected mice at a final concentration of 1.5 × 10^7 cells/ml for each source. Cells from uninjected and SEA injected mice were treated with a 10^{-4} dilution of monoclonal anti-Thy-1.2 antibody plus complement where indicated prior to mixing. All cultures were stimulated with 0.5 μg SEA/ml. Spleen cell cultures: ●, 24-h SEA spleen cells + virgin spleen cells; ○, 24-h SEA spleen cells treated with anti-Thy-1.2 antibody + virgin spleen cells; ■, virgin spleen cells; □, virgin spleen cells treated with anti-Thy-1.2 antibody; ▲, 24-h SEA spleen cells; △, 24-h SEA spleen cells treated with anti-Thy-1.2 antibody. Assays were carried out in duplicate and coefficients of variation were less than 20%.

Regulation of IFNγ production by spleen cells from mice injected with SEA

The regulation of the IFN response to SEA was examined by mixing spleen cells from mice injected 24 h previously with SEA (24-h SEA spleen cells) with cells from uninjected mice (virgin spleen cells). Spleen cells from uninjected mice, when stimulated in vitro with SEA, produced increasing levels of IFN as a function of time (Fig. 2). Spleen cells from mice injected 24 h previously with SEA produced a declining IFN response when stimulated in vitro, most noticeable on days 2 and 3. The mixture of virgin spleen cells and 24-h SEA spleen cells showed a response similar to that of 24-h SEA spleen cells alone. The response was 10-fold less than that of the same concentration of virgin spleen cells alone. Obviously, the cells obtained from mice injected with SEA had a suppressive effect on the IFN response of virgin spleen cells to in vitro stimulation with SEA.

Effect of anti-Thy-1.2 antibody treatment on the suppressor cell activity

The suppressor cell preparation was treated with a 10^{-4} dilution of monoclonal anti-Thy-1.2 antibody and complement in order to determine if the effector cell was a T cell. Mixtures of anti-Thy-1.2 antibody-treated 24-h SEA spleen cells and virgin spleen cells gave high SEA-induced IFNγ responses over 3 days, indicating that the treatment eliminated the suppressor cell effects (Fig. 2). Anti-Thy-1.2 antibody-treated virgin spleen cells failed to produce IFN when stimulated in vitro by SEA. Surprisingly, anti-Thy-1.2 antibody-treated 24-h SEA spleen cells were relatively potent producers of IFN with a peak interferon titer on day 2. Thus, anti-Thy-1.2 antibody treatment that blocked the response of virgin spleen cells enhanced the response of spleen cells from SEA-injected mice. These data suggest that induction of IFNγ in virgin spleen cells requires a T-cell population carrying a high density of the Thy-1 antigen. Spleen cells from mitogen-primed mice appeared to contain a population of cells with a relatively high density of Thy-1 antigen that suppressed IFN production. Finally, 24-h SEA spleens were found to contain another cell population which lacked the Thy-1 antigen or contained only a low density of it, and which was able to produce relatively large amounts of IFN in the absence of the inhibitory cell population.

To further examine the properties of the spleen cells from SEA-primed mice that produced IFNγ, 24-h SEA cells were treated with anti-Thy-1.2 antibody at a 10^{-3} dilution, i.e., at a 10-fold higher concentration than in the previous experiments. The results are presented in Fig. 3. The patterns of the IFN response were generally the same as in

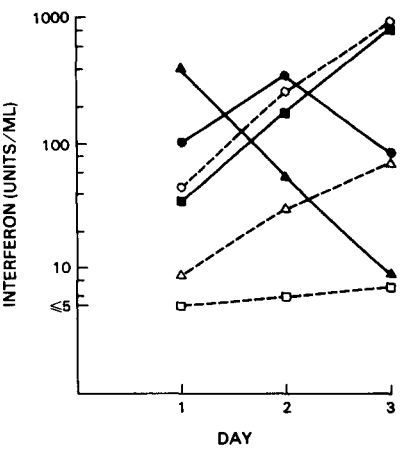

Fig. 3. Regulation of IFNγ production by spleen cells from mice injected with SEA. Conditions were exactly the same as in Fig. 2, except that the anti-Thy-1.2 antibody concentration was increased 10-fold to a 10^{-3} dilution for treatment of cells. Spleen cell cultures: ●, 24-h SEA spleen cells + virgin spleen cells; ○, 24-h SEA spleen cells treated with anti-Thy-1.2 antibody + virgin spleen cells; ■, virgin spleen cells; □, virgin spleen cells treated with anti-Thy-1.2 antibody; ▲, 24-h SEA spleen cells; △, 24-h SEA spleen cells treated with anti-Thy-1.2 antibody. Assays were carried out in duplicate and coefficients of variation were less than 20%.

Fig. 2, except that the response of the anti-Thy-1.2 antibody-treated spleen cells from SEA-primed mice was relatively low. Thus, treatment with the higher concentration of anti-Thy-1.2 antibody blocked the IFN response in contrast to enhancement obtained with the lower concentration. Thus, this IFN-producing cell (after in vivo mitogen stimulation) appeared to contain a low density of the Thy-1 antigen and was probably a T cell. Alternatively, it is possible that the anti-Thy-1.2 treatment simply reduced the number of T cells in the suppressor cell population in a non-selective fashion. If this were so, the loss of suppression of virgin spleen cells and production of IFN by the suppressor cell population should be observed with lower concentrations of untreated suppressor cells. Lower, non-suppressive concentrations of the suppressor cell population (0.1–1.0 $\times 10^6$ cells/ml) that were not treated with anti-Thy-1.2 antibody did not produce immune interferon. Thus, the above responses could not be mimicked by simply diluting the suppressor cell population, which suggests that the anti-Thy-1.2 antibody effects were not simply 'dilution' effects on the T-cell population.

Mitogen stimulation of spleen cell cultures that were treated with different concentrations of anti-Thy-1.2 antibody

The concentrations of anti-Thy-1.2 antibody that were used in the experiments of Figs. 2 and 3 were also used to treat virgin spleen cells that were subsequently stimulated with B- and T-cell mitogens. The objective of these experiments was to determine whether the 10^{-4} and 10^{-3} dilutions of the anti-Thy-1.2 antibody had differential effects on mitogenesis. The results are presented in Table 2. The antibody treatment blocked the mitogenic responses to T-cell mitogens. Anti-Thy-1.2 antibody had no effect at any concentration on the response to the B-cell mitogen LPS, thus suggesting that B cells were not affected. The 10^{-4} dilution of anti-Thy-1.2 antibody was essentially as effective as the 10^{-3} dilution in suppressing the T-cell response to concanavalin A (Con A), phytohemagglutinin-P (PHA-P), and SEA. Thus, no obvious or overt differential concentration effect was observed in the effect on T-cell mitogenesis. The mitogenic activity of unstimulated cultures that were treated with the 10^{-3} dilution of antibody was less than that of similar cultures treated with a 10^{-4} dilution.

Kinetics of disappearance of IFNγ from virgin and 24-h SEA spleen cell cultures

In order to determine whether the apparent inhibitory effect of 24-h SEA spleen cells on the IFN response of virgin spleen cells was due to blocking of production of IFN or to enhanced destruction, IFNγ was added to virgin and to 24-h SEA cultures and the kinetics of its disappearance was followed. As seen in Table 3, the IFN activity disappeared from both cultures at approximately the same rate, suggesting that 24-h SEA spleen cells block IFN production rather than enhancing its destruction or inactivation.

TABLE 2

Mitogen stimulation of C57B1/6 mouse spleen cells that were treated with different concentrations of monoclonal antibodies to Thy-1.2 alloantigen[a]

Exp.	Dilution of anti-Thy-1.2	Mitogen[b]	c.p.m. ± S.D.
1	10^{-4}	LPS	179,817 ± 430
	10^{-4}	Con A	9,565 ± 150
	10^{-4}	PHA-P	6,260 ± 1,406
	10^{-4}	–	6,252 ± 527
	10^{-3}	LPS	261,032 ± 7,929
	10^{-3}	Con A	11,494 ± 139
	10^{-3}	PHA-P	8,048 ± 1,690
	10^{-3}	–	2,505 ± 171
	–	LPS	199,034 ± 19,166
	–	Con A	112,074 ± 5,220
	–	PHA-P	91,557 ± 1,950
	–	–	9,541 ± 5,899
2	10^{-4}	SEA	5,227 ± 790
	10^{-4}	–	2,015 ± 185
	10^{-3}	SEA	4,495 ± 282
	10^{-3}	–	3,785 ± 375
	–	SEA	22,464 ± 2,394
	–	–	4,889 ± 234

[a] Spleen cells, 5 × 10^7/ml, plus anti-Thy-1.2 antibody were incubated at 37°C for 1 h with guinea pig complement. The cells were washed 3 times and diluted to a concentration of 1.5 × 10^7 cells/ml in RPMI culture medium. Cells were stimulated three days with the indicated mitogens. ^{3}H-TrD, 2 μCi, was added on day 2 and the cells were harvested and counted on a Beckman LS 9000 liquid scintillation counter.

[b] LPS, 100 μg lipopolysaccharide/ml; Con A, 4 μg concanavalin A/ml; PHA-P, 10 μg phytohemagglutinin-P/ml; SEA, 0.5 μg staphylococcal enterotoxin A/ml.

TABLE 3

Kinetics of disappearance of IFNγ from normal and 24-h SEA spleen cell cultures[a]

Day	Interferon (units/ml)	
	Normal cultures	24-h SEA cultures
1	22	50
2	7	3
3	3	3

[a] IFNγ, 200 units/ml final concentration, was added to cultures containing a final concentration of 1.5 × 10^7 cells/ml. Residual activity was measured at the indicated days. SEA activity in the interferon was neutralized by adding excess anti-SEA antibody. Assays were carried out in duplicate and coefficients of variation were less than 20%.

DISCUSSION

Spleen cells from mice injected intravenously with the T-cell mitogen SEA produce IFNγ when put in culture shortly (2 h) after injection. The production is enhanced when the cells receive additional stimulation with SEA in vitro. Spleen cells obtained from mice 24 h after SEA injection (24-h SEA spleen cells) did not produce IFN. Restimulation with SEA in vitro enhanced the response on day 1 in culture, but by day 3 the IFN activity was 60-fold less than that of spleen cells from uninjected mice.

Mixing experiments using 24-h SEA spleen cells and cells from the uninjected mice suggested that 24-h SEA spleen cells contain a population of cells with inhibitory effects on the IFN response of virgin spleen cells. This suppressive effect could be abrogated by pretreating the 24-h SEA spleen cells with a relatively high dilution of monoclonal antibody to the Thy-1.2 T-cell alloantigen. Treatment of virgin spleen cells with anti-Thy-1.2 antibody under the same conditions resulted in the complete loss of ability of these cells to produce IFN. This was expected since T cells are known to be the source of IFNγ [5]. The IFN response of anti-Thy-1.2 antibody-treated 24-h SEA spleen cells was high over the 3-day incubation period. This was surprising since T cells would also be involved in IFNγ production here and should therefore be suppressed in that response by anti-Thy-1.2 antibody. Increasing the concentration of anti-Thy-1.2 antibody 10-fold did indeed suppress the IFN response of the 24-h SEA spleen cells relative to the appropriate controls. No obvious differences were detected in the ability of the two concentrations of anti-Thy-1.2 antibody to suppress blastogenesis in response to T-cell mitogens. IFN added to either virgin or 24-h SEA spleen cells lost activity with similar kinetics, indicating that 24-h SEA cells exert their suppressive effects through a block of IFN induction as opposed to enhancing its destruction or inactivation. Since 0.01–10 μg SEA induce spleen cells to produce IFNγ to the same extent [11], it is unlikely that injection of 5 μg SEA into mice resulted in 'exhaustion' of spleen cells to further stimulation by 0.5 μg SEA in culture.

Our findings can be used to construct a model of the cellular events involved in the induction and regulation of IFNγ production. According to this model induction of virgin spleen cells for IFNγ production involves a T cell(s) with a relatively high density of the Thy-1.2 antigen. Upon stimulation this cell evolves along two paths: an IFN-producing cell of relatively low Thy-1.2 antigen density, and a suppressor (or feedback inhibitor) cell with a relatively high density of the Thy-1.2 antigen. Temporally, the IFN-producing cell precedes the suppressor cell in functional development, but is regulated by the suppressor cell. The IFN-producing cell (assuming sensitivity to mitogenesis) may comprise a small fraction of the T-cell population, since gross differences in suppression of T-cell mitogenesis were not observed by treatment with 10-fold differences in anti-Thy-1.2 antibody concentration, even though IFN production (relative to controls) was significantly affected. We realize that more complex models can be evoked, such as a separate cell with a high density of Thy-1.2 antigen as the precursor for the suppressor cell, or a helper cell population of high density of Thy-1.2 for an IFN-produc-

ing cell of low density of Thy-1.2 antigen. Modifications of the above model will be made as dictated by the results of future experiments.

There are additional considerations. Firstly, the suppressor cell population was found in mouse spleens when tested at 72 h after SEA injection (data not shown). Preliminary experiments show that Con A and PHA-P induce the same cellular events as SEA (B. Torres and H.M. Johnson, unpublished data). Secondly, IFN may not be the inducer of the suppressor cell population, since cultures treated with interferon for 3 days did not suppress untreated cells in mixing experiments. Preliminary experiments suggest that the suppressor cell can be induced in vitro, thus ruling out a shift in the cell population in the spleen to explain the in vivo stimulations (B. Torres and H.M. Johnson, unpublished data). Thirdly, our findings are consistent with in vivo studies where mice were sensitized by infection with *Mycobacterium bovis* (strain BCG) [19]. When challenged later with old tuberculin (OT), they produced high levels of IFN. Such mice were refractory to further IFNγ production for about two weeks when restimulated with OT.

Recently, a T-suppressor cell population(s) has been shown to inhibit production of the lymphokine macrophage migration inhibitory factor [3, 7, 17]. These data coupled with those of the present report provide strong evidence for a T-suppressor cell regulatory mechanism in lymphokine production.

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