Differential Regulation of the Immune Response to SRBC by Monoclonal Antibodies to Interferon-γ (42889)

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Abstract. **Three monoclonal antibodies against different epitopes of interferon-γ (IFN-γ) were used to assess its role as a normal immunomodulatory molecule. Two of these antibodies were able to reduce significantly the primary antibody response to sheep erythrocytes in an *in vitro* culture system. One of these two antibodies has been reported to suppress both the antiviral and macrophage activation factor activities of IFN-γ by binding to its carboxyl terminus. These findings indicate that IFN-γ is an important lymphokine for the maximum expression of the immune response and that it acts via the carboxyl terminus of the molecule. This antibody suppressed the immune response only when added at the initiation of culture, suggesting that the action of IFN-γ is on an early component of the response. The third monoclonal antibody, which binds to the amino end of IFN-γ, did not suppress the *in vitro* response. However, it was able to block the effects exerted by an immunosuppressive dosage of exogenous IFN-γ on *in vitro* antibody production. These results indicate that the immunosuppression induced by the addition of IFN-γ to a primary antibody response and the role that it plays in that response are mediated through different sites on the molecule and, therefore, probably by different mechanisms.** [P.S.E.B.M. 1989, Vol 191]

Interferon-γ (IFN-γ) has been proposed as an important regulator of immunologic reactions, mainly because it has been shown to be capable of modulating an immune response when introduced during that response (1–5). The effects of exogenous IFN-γ may be either suppressive or enhancing, depending on how and when it is administered (1–5). Nonetheless, there have been few studies that have demonstrated that IFN-γ has an intrinsic immunomodulatory role, or that IFN-γ is even produced during a primary response to antigen (6–8). The difficulty in detecting IFN-γ during primary immune responses may be due to production of very low levels or to production that merely keeps pace with binding and/or utilization of IFN-γ during the response.

In the work to be described, three monoclonal antibodies which block the macrophage-activating capability and/or the antiviral activity of IFN-γ were tested for their ability to modify the *in vitro* immune response to sheep erythrocytes (SRBC) (9, 10). Such modification would indicate that IFN-γ is a functional component of the normal immune response to antigen. It indeed has been possible to demonstrate, by use of these monoclonal antibodies, that IFN-γ is necessary for a maximal response to antigen and further that the carboxyl terminus is important to that effect. In addition, there appears to be a difference in the ability of each monoclonal antibody to block suppression induced by exogenous IFN-γ and the enhancement by endogenous IFN-γ, suggesting that these two functions may be mediated by different sites on the IFN-γ molecule.

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Materials and Methods

Animals. Female C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN) or were bred from Harlan stock at the University of Louisville. These mice provided the spleen cells for all *in vitro* immunizations. Mice were kept in an AALAC accredited facility under the care of a veterinarian and fed mouse chow and water *ad libitum.*

Antigens. SRBC were obtained by periodic bleedings of a single sheep (Gibco Laboratories, Madison, Wisconsin) and stored in Alsever's solution at 4°C.

Primary Antibody Response Culture. To culture mouse spleen cells for a primary antibody response to SRBC, a modification of the Mishell-Dutton culture

was used (11). A single-cell suspension was prepared in primary antibody response medium (PARM), which consisted of 1× minimal essential medium with Hanks' salt solution (Gibco Laboratories, Grand Island, NY), 2 m*M* glutamine, 25 m*M* sodium pyruvate, 1× nonessential amino acids (Flow Laboratories, Rockville, MD), antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml Fungizone), 2×10^{-5} *M* 2-mercaptoethanol, 9 m*M* sodium bicarbonate, 20 m*M* Hepes buffer (Research Organics, Cleveland, OH), 5% fetal calf serum (Lot #25F-0561, Sigma Chemical Co., St. Louis, MO), and 0.5% normal syngeneic mouse serum. Spleen cells were cultured in PARM at a concentration of 5×10^6 cells/ml with or without 0.025% SRBC, IFN-γ, or monoclonal antibody to IFN-γ. Cultures were maintained in 200-μl volumes in 96-well microtiter plates at 37°C in a 5% CO_2 in an air atmosphere for 4 days, at the end of which time the cells were harvested and the number of antibody plaque-forming cells (PFC) per 10^6 viable spleen cells was determined. In some cultures, supernatants were collected during culture to test for levels of antiviral activity.

IFN-γ and Antibodies to IFN-γ. Purified recombinant IFN-γ was a gift from Genentech Corp. (San Francisco, CA). The hamster anti-murine IFN-γ antibodies H1.5 and H22 were generously provided as protein A-purified monoclonal antibody preparations by Dr. R. Schreiber of Washington University, St. Louis, MO (9). The rat monoclonal antibody R4-6A2 containing culture supernatant was a generous gift from Dr. E. Havell of the Trudeau Institute, Saranac Lake, NY (10).

Interferon Assay. The interferon titer of samples was determined by a vesicular stomatitis virus plaque reduction assay on L929 cells (12). Cells were stained with 0.5% crystal violet in 40% ethanol and plaques were counted with the aid of a dissecting microscope (13).

Results

Endogenous IFN-γ Production during *In Vitro* Culture of Murine Spleen Cells. Normal spleen cells cultured with and without SRBC produced low levels of antiviral activity that increased with time in culture (Table I). This activity was inhibited by antibody to IFN-γ (data not shown). Supernatants of cultures with SRBC contained less demonstrable IFN-γ than those without, particularly in the early stages of the response. Binding of IFN-γ to cells undergoing a primary response in culture could account for this observation and would be consistent with the notion that IFN-γ is a component of the response.

Effects of Anti-IFN-γ Monoclonal Antibodies on the Ability of IFN-γ to Inhibit a Primary Antibody Response to SRBC. Exogenous IFN-γ has been shown to modulate primary antibody responses to SRBC both *in vitro* and *in vivo* (1–5). It was therefore of interest to determine whether monoclonal antibodies could block the effects of added IFN-γ. Two of the antibodies used bind to different epitopes on the IFN-γ molecule and differentially modulate functions of IFN-γ (9). Monoclonal antibody H1.5 binds to the amino end of IFN-γ and blocks only its antiviral activity, whereas H22 binds to the carboxyl end of the molecule and can block both antiviral activity and macrophage activation by IFN-γ. When IFN-γ was added to a Mishell-Dutton-type culture, the antibody plaque-forming cell response in that culture was suppressed, as seen in Table II. In contrast to H22, monoclonal antibody H1.5 did block the suppression induced by exogenous IFN-γ, indicating that this effect is mediated by the amino end of IFN-γ.

Ability of Antibodies to IFN-γ to Modulate the Primary Antibody Response to SRBC. To determine whether naturally occurring IFN-γ is an important

Table I. Production of IFN-γ during Culture of Normal Mouse Spleen Cells for Induction of an *In Vitro* Immune Response to SRBC

Duration of culture (days)[a]	*n*	SRBC added	Units of IFN-γ ± SEM[b]
1	5	−	7 ± 1
1	5	+	8 ± 2
2	6	−	7 ± 2
2	5	+	4 ± 2
3	5	−	12 ± 5
3	5	+	4 ± 1
4	3	−	41 ± 27
4	5	+	10 ± 4
5	7	−	42 ± 7
5	6	+	28 ± 4

[a] Cells were cultured in PARM for induction of a primary antibody response to SRBC and supernatants were collected at 24-hr intervals after the initiation of culture.
[b] Culture supernatants were titered for interferon by a vesicular stomatitis virus plaque reduction assay.

Table II. The Primary Antibody Response to SRBC *In Vitro*: Effects of Two Monoclonal Antibodies to IFN-γ on Spleen Cell Culture with or without IFN-γ[a]

Antibody added	Recombinant IFN-γ added (10 units/ml)	PFC/10^6 spleen cells ± SEM[b]
None	−	1410 ± 258
None	+	691 ± 119
H1.5	−	1120 ± 114
H1.5	+	1369 ± 423
H22	−	499 ± 153[c]
H22	+	508 ± 73[c]

[a] Cells were cultured in PARM for 4 days with or without IFN-γ and/or monoclonal anti-IFN-γ antibodies H1.5 or H22.
[b] Data presented were the result of three experiments in each of which all variables were tested on spleen cells from a single mouse.
[c] The difference between untreated control cells and those cultured with H22 antibody with or without IFN-γ was statistically significant at $P < 0.05$ by Student's *t* test.

component of the primary *in vitro* immune response, antibodies to IFN-γ were added to murine spleen cells cultured with SRBC. In these experiments, H22 and H1.5 antibodies were used at 0.2 and 2.0 μg/ml, respectively, which was sufficient to inhibit 10 units/ml IFN-γ. Although H22 antibody inhibited the normal primary antibody response to SRBC, the H1.5 antibody had no effect (Table II). The inability of H1.5 antibody to affect these cultures indicates that the effect of H22 antibody is not due to a nonspecific effect of hamster immunoglobulin. A third monoclonal antibody, R4-6A2, also binds to IFN-γ and can inhibit antiviral and macrophage activation factor (MAF) activity (10). R4-6A2, at a 1/10 dilution, also decreased the plaque response to SRBC (Table III), although about 8–10 times more was required to produce this effect than that required to neutralize the antiviral activity of IFN-γ. Additions of similar concentrations of myeloma culture supernatants to these cultures had no effect on the outcome of the culture (data not shown).

Time of IFN-γ Action. In order to determine at what stage of the immune response IFN-γ is necessary, antibody H22 was added at intervals from the time of addition of antigen (Table IV). Addition of H22 antibody in combination with antigen suppressed the response, while addition at 24 hr or later did not produce a significant suppression. Thus, IFN-γ appears to act on an early component of the immune response.

Table III. The Primary Antibody Response to SRBC *In Vitro*: Effects of R4-6A2, a Monoclonal Antibody to IFN-γ

Antibody added	PFC/10^6 spleen cells ± SEM[a]
None	1131 ± 118
R4-6A2	608 ± 127[b]

[a] Data presented were the results of three experiments in which all variables were tested on spleen cells from a single mouse.
[b] The difference between the antibody treated group and the control group was statistically significant at $P < 0.05$ by Student's *t* test.

Table IV. The Primary Antibody Response to SRBC *In Vitro*: Kinetics of the Anti-IFN-γ Antibody-Induced Suppression

Time after immunization[a] (hr)	*n*	Treatment	
		Medium	H22 antibody
		PFC/10^6 spleen cells ± SEM	
0	4	1369 ± 389	936 ± 337[b]
24	4	1462 ± 386	1120 ± 304
48	4	1148 ± 303	1032 ± 338

[a] Fresh spleen cells were isolated and cultured with 0.025% SRBC in PARM for *in vitro* immunization.
[b] The difference between cells cultured with antibody and those only cultured with medium was statistically significant at $P < 0.05$ by Student's *t* test.

Discussion

Numerous investigators have demonstrated that exogenous IFN-γ is capable of enhancing or suppressing an immune response to SRBC (1–5). While most such studies have been done with crude preparations of lymphokines containing IFN-γ, Johnson and Torres (5) have shown that recombinant mouse IFN-γ can suppress this response as well. In the present work, 10 units/ml of recombinant murine IFN-γ added at the same time as antigen suppressed the response by about 50%. Although this further confirms that IFN-γ can function as an immunomodulatory agent, the current question is whether intrinsic IFN-γ acts as a physiologic mediator of the immune response (4).

In order to examine further the role of IFN-γ in a primary antibody response, monoclonal antibodies were used in attempts to inhibit its function. Antibody H1.5 completely blocked the IFN-γ-induced suppression of the response, but had no effect on the control response, indicating that the amino end of the molecule was involved in suppression of the response by exogenous IFN-γ. The H22 antibody could not block suppression by IFN-γ, but reduced the control response significantly, as did the R4-6A2 antibody. The ability of antibodies to IFN-γ to alter an otherwise normal response to antigen strongly suggests that IFN-γ is a component of the primary antibody response, although it may only be necessary for maximizing such a response. The inability of H1.5 antibody to affect the response to SRBC suggests that the effect of the other antibodies on the response is not due to nonspecific effects of foreign immunoglobulin or antigen-antibody complexes, but is due to the binding of the antibody to a specific site on the IFN-γ molecule. The inhibition of the response by H22 antibody indicates that a site on the 35-amino acid carboxyl terminal end of the IFN-γ is necessary for it to function in the *in vitro* primary antibody response. This is supported by an independent study by Langford *et al.* (8) in which they demonstrate that a polyclonal antibody to a 9-amino acid peptide from the carboxyl terminus of IFN-γ is able to inhibit the primary antibody response to SRBC *in vivo*. Both the antibody of Langford *et al.* (8) and H22 inhibit MAF activity of IFN-γ as well as its antiviral activity (9).

Suppression of the primary antibody response to SRBC by antibodies to IFN-γ provides evidence that there is indeed a physiologic role for IFN-γ in that response. The low levels of detectable IFN-γ in supernatants of control immunized cultures, at a time when the addition of antibody to IFN-γ has a significant suppressive effect on the response, suggest that IFN-γ may be rapidly bound by cells of the immune response and therefore may not be readily detectable in supernatants of these cultures. Schreiber *et al.* (9) have reported that binding of IFN-γ to macrophages is inhib-

ited by H22 antibody, suggesting that the H22 epitope is in the macrophage receptor binding site. Since they did not examine the binding of IFN-γ to any other cell type, it is not clear if there is binding of IFN-γ to T and B cells through this epitope. The differential action of H1.5 and H22 antibody suggests that the mechanism of action of exogenous IFN-γ in suppressing a primary response and that of endogenous IFN-γ in maximizing a normal response are mediated by different sites on the IFN-γ molecule (9), which bind to different cellular receptors.

The data presented here and that of Langford *et al.* (8) suggest that endogenous IFN-γ is an important component in the induction of an optimal primary antibody response. This effect of IFN-γ probably is not on the B cell, because its known effects on the B cell are primarily suppressive or late or involve selective class switching, whereas in this study, endogenous IFN-γ has been found to have an early enhancing effect on the primary IgM antibody response (2, 15–18). The T cell cannot be ruled out as the site of the intrinsic immunoregulation by IFN-γ (19, 20); however, there is no evidence to directly link the epitopes on the IFN-γ molecule which are blocked by anti-IFN-γ antibody with T cell regulation by IFN-γ. In contrast, the H22 antibody has been shown to block the effects of IFN-γ on macrophages, including activation of macrophages and induction of surface Ia antigen (9, 21–24). The induction of Ia antigens on macrophages would facilitate antigen presentation and the consequent upregulation of the immune response. Vogel *et al.* (25) have reported that the inhibition of IFN-γ-induced Fc receptors requires 10–50 times more R4-6A2 antibody than inhibition of Ia antigen expression, again suggesting that there are different functional epitopes on the IFN-γ molecule, and that this antibody may selectively disrupt Ia antigen expression on the macrophage at the dose used. Additionally, the quantitative difference in the ability of R4-6A2 to block antiviral activity in L cells and the primary antibody response correlate with data by Spitalny and Havell (10) that show a similar difference in the inhibition of antiviral activity and MAF. Vogel *et al.* (25) demonstrate that this difference is due to a difference in the ability of anti-IFN-γ to inhibit IFN-γ activity on L cells as compared with macrophages. The ability of an antibody directed toward a single epitope on the interferon molecule to block MAF and to inhibit a primary antibody response suggests that the macrophage is the site of the IFN-γ action. The inability of the monoclonal antibody H1.5 to inhibit either MAF or to depress a primary antibody to SRBC also supports this possibility (9).

IFN-γ is a molecule with multiple functions. Its role as an immunomodulatory agent has been studied for a number of years. However, it is clear that the understanding of its physiologic function is still far from complete (4). Data presented in this study provide evidence for IFN-γ production during the cultivation of normal spleen cells and for an early role for IFN-γ in maximizing a primary antibody response to SRBC *in vitro*. The data support a model in which endogenous IFN-γ acts on the macrophage, probably to induce Ia antigens, increase antigen uptake, and/or to increase production of IL-1, thus upregulating the ongoing response. The inability of antibody to IFN-γ to completely block the response may be due to a level of endogenous activity by the macrophage sufficient to produce a partial response or to a simultaneous activation of other pathways that partially fulfill this function.

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Erratum

The names and addresses of the following members were inadvertently omitted from the 1988 Membership Directory (published in the December 1988 issue of *PSEBM*):

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