Interferons Exhibit Temporally Distinct Regulation of Two Bovine Macrophage Fc Receptors

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Interferon (IFN)-induced modulation of two distinct types of Fc receptors (FcR) on bovine bone marrow culture-derived macrophages was quantified by flow cytometry. We have established the presence of separate FcR for monomeric and aggregated IgG on these cells, equivalent to the FcRI and FcRII of mice, respectively. These two kinds of FcR differed in protease sensitivity, hierarchy of preferential binding of immunoglobulin subclass, and cross-inhibition. Treatment of macrophages with either recombinant bovine IFN-gamma (rBolIFNγ) or rBolFNα1 produced a dose-dependent increase in the expression of both types of FcR; however, expression of the FcR for aggregated IgG was increased a full 24 h prior to that for monomeric antibody. Further, expression of the FcRII-equivalent declined substantially by 48 h of IFN exposure, while the presence of the FcRI-equivalent receptor was still enhanced. Finally, low doses (1 unit/ml) of either rBolIFNγ or rBolFNα1 failed to alter FcR expression, yet mixtures of IFNs at this concentration were able to potentiate FcR expression. This is the first time that a differential time course of induction of FcR types by IFNs and the effects of mixtures of IFNs on FcR expression has been described in any species.

Key words: interferon-gamma, interferon-alpha, flow cytometry

INTRODUCTION

Expression of FcR on the surface of macrophages is integral to the function of the immune system. Interaction of opsonized pathogens with macrophage FcR initiates cellular defense mechanisms including phagocytosis, microbicidal activity, antibody-dependent cell-mediated cytotoxicity, and the release of lysosomal enzymes and reactive oxygen intermediates. In the mouse, two separate types of FcR, one for monomeric and one for aggregated IgG, have been described [9,18]. In addition to the difference in the polymerization of antibody required for binding, these FcR types also differ with respect to trypsin sensitivity [4,18] and immunoglobulin subclass specificity [4,9,20] as well as differential sensitivity of IgG2a receptors to low temperatures and cytchalasin [4]. FcR have also been demonstrated on the surface of bovine mononuclear phagocytes [15]; however, their characterization has been limited to demonstration of FcR for IgG1 and IgG2 subclasses by using binding of antibody-coated erythrocytes [10,12,16]. The present study will extend these earlier investigations by characterizing the bovine FcR in detail. In addition, the effect of IFNs, used alone and in mixtures, on the expression of bovine FcR will be demonstrated. Our findings indicate that two distinct FcR types are present on bovine macrophages and that they can be independently regulated by IFNs. We will also show that the expression of FcR is potentiated by mixtures of gamma and alpha IFNs, compared to either type used alone.

MATERIALS AND METHODS

Tissue Culture Medium and Miscellaneous Reagents

Modified Eagle's minimal essential medium (HMEM) was prepared from a powdered mix (Auto-POW MEM) and supplemented with sodium bicarbonate (2 mg/ml), glutamine (2 mM) (both from Flow Laboratories (McLean, VA), injectable penicillin G potassium (100 u/ml), injectable streptomycin sulfate (100 u/ml) (both from Pfizer), and 15 mM HEPES (Sigma Chemical Co., St. Louis, MO). Fetal bovine serum (FBS) was obtained from Sterile Systems (Logan, UT), and horse serum (HS) was obtained from Flow Laboratories (McLean, VA). rBolIFNα1 and rBolFNγ were kindly supplied by Genentech Incorporated (South San Francisco, CA) and

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Ciba-Geigy (Basel, Switzerland). The specific activity of the rBoIFNα was \(2 \times 10^6\) /mg protein and that of the rBoIFNα1 was \(1.3 \times 10^7\) /mg protein when titrated in a plaque inhibition assay on Madin-Darby bovine kidney (MDBK) cells challenged with vesicular stomatitis virus. All tissue culture media, sera, and IFNs used in this study were negative for endotoxin, as determined by assay with *Limulus* amebocyte lysate (Associates of Cape Cod, Woods Hole, MA) at a sensitivity level of 0.07 ng/ml. Trypsin (Sigma, St. Louis, MO) type III from bovine pancreas exhibited 37 u/mg protein, as determined by the hydrolysis of p-toluenesulfonyl-L-arginine methyl ester/minute. Fluorescein isothiocyanate (FITC)-conjugated and unconjugated bovine IgG were purchased from Cooper Biomedical, Inc. (Malvern, PA). The FITC-IgG had a fluorescein/protein ratio of 6.57 μg/mg. Bovine IgG1, IgG2a, and IgG2b were generously provided by Dr. Michael Boyle, University of Florida. Dichlorotriazinyl amino fluorescein-conjugated F(ab')2 fragment of goat anti-bovine IgG directed against the light chains of all bovine immunoglobulins was purchased from Jackson ImmunoResearch Lab. (West Grove, PA).

**Bovine Bone Marrow Culture-Derived Macrophages**

Nucleated cells were obtained from the red marrow of the long bones of newborn Holstein calves and immediately frozen in liquid nitrogen in HMEM containing 8% dimethyl sulfoxide (Sigma) and 25% FBS. Cells were thawed into supplemented growth medium containing 10% FBS, 5% HS, and 15% fibroblast-conditioned medium which had been collected from 4 d, confluent monolayers of primary bovine fibroblasts and filter sterilized. The proliferating bone marrow cells were first carried in plastic tissue culture flasks (Corning) for two successive 24 h periods at a density of \(2 \times 10^5\) cells/cm² in order to deplete contaminating adherent stromal cells. Nonadherent cells remaining in the cultures were then transferred to bacterial grade Petri dishes and cultured for an additional 8 to 10 d. Cultures were fed with 5 ml fresh medium every 3 d and used 72 h after their last feeding. By 7 d of culture, greater than 95% of the recovered adherent cells exhibited macrophage morphology, and virtually all of these cells were strongly esterase-positive by day 10.

**FcR Binding of Immunoglobulin Subclasses**

Monomeric or heat-aggregated immunoglobulins of subclasses IgG1, IgG2a, and IgG2b were each prepared as described above. One million cells were incubated with 1,000 u/ml of rBoIFNα for 24 or 48 h to examine expression of FcR for aggregated and monomeric subclasses of IgG, respectively. Cells were labelled for 45 min at 4°C with a mixture of \(5 \times 10^{-7}\) M of either IgG1 or IgG2a or IgG2b and a \(5 \times 10^{-7}\) M fluorescein-conjugated F(ab')2 fragment of goat anti-bovine IgG reactive with light chains of bovine immunoglobulins. The latter was specific for the F(ab')2 fragment of the bovine subclasses used in this study. There was no detectable binding of the fluoresceinated goat F(ab')2 fragment used alone. Nonspecific binding was quantified for each subclass as described above.
RESULTS

Characterization of Bovine FcR

Protease sensitivity of the FcR for monomeric or heat-aggregated IgG. Specific binding of monomeric IgG to its FcR is represented by the difference in MFC between cells labelled with FITC-IgG alone and cells labelled with FITC-IgG in the presence of a 100-fold excess of unconjugated IgG. Binding to the FcR for monomeric antibody could be eliminated by trypsin treatment (Table 1). In contrast, not only was the FcR for heat-aggregated IgG resistant to proteolytic activity, its capacity to bind complexes was enhanced.

Cross-inhibition of binding of FITC-IgG. Monomeric FITC-IgG could be effectively blocked from binding to the macrophage surface by a 100-fold excess of either monomeric or heat-aggregated IgG (Table 2). Aggregated antibody could, therefore, interact with the FcR which binds monomeric IgG, as well as the one that bound complexes. By comparison, specific binding of heat-aggregated FITC-IgG to its FcR could only be

| TABLE 1. Protease Sensitivity of FcR on Bovine Bone Marrow Culture-Derived Macrophages |
|--------------------------------------|-----------------|-----------------|
|                                      | Mean fluorescence channel* |
|                                      | Without trypsin | After trypsin   |
| Autofluorescence                     | 2.15            | 2.11            |
| Monomeric FITC-IgG                   | 5.31            | 3.03            |
| Monomeric FITC-IgG + unconjugated monomeric IgG | 2.27            | 3.06            |
| Heat-aggregated FITC-IgG             | 12.75           | 17.25           |
| Heat-aggregated FITC-IgG + unconjugated heat-aggregated IgG | 6.73            | 8.79            |

*The results are expressed as mean fluorescence channel (MFC) from one of four replicate experiments. Equivalent percent changes in MFC were observed upon trypsinization in each trial. One × 10^6 bone marrow macrophages were treated in 1 ml of medium alone or medium containing trypsin (1 mg/ml) for 30 min at 37°C. No decrease in viability of macrophages was observed following this procedure, as determined by trypan blue dye exclusion. Cells were labelled with 5 × 10^{-7} fluorescein-conjugated IgG (FITC-IgG) in the presence of PBS or a 100-fold excess of unconjugated IgG.

Fig. 1. Time course of rBoIFNγ and rBoIFNα1 effects on macrophage expression of FcR for monomeric bovine IgG. Bovine bone marrow culture-derived macrophages were treated for the indicated times with either medium or 1,000 u/ml of either IFN. Representative data from one of four replicate experiments are presented as single-parameter histograms of green fluorescence, indicating the intensity of binding of the FITC-IgG. The extent of nonspecific binding in each sample was identical within time points.
TABLE 2. Inhibition of Binding of FITC-IgG to Bovine Bone Marrow Culture-Derived Macrophages

<table>
<thead>
<tr>
<th></th>
<th>MFC*</th>
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<tbody>
<tr>
<td>Autofluorescence</td>
<td>2.21</td>
</tr>
<tr>
<td>Monomeric FITC-IgG</td>
<td>6.49</td>
</tr>
<tr>
<td>Monomeric FITC-IgG + Monomeric IgG</td>
<td>3.87</td>
</tr>
<tr>
<td>Monomeric FITC-IgG + heat-aggregated IgG</td>
<td>2.94</td>
</tr>
<tr>
<td>Heat-aggregated FITC-IgG</td>
<td>29.08</td>
</tr>
<tr>
<td>Heat-aggregated FITC-IgG + heat-aggregated IgG</td>
<td>15.11</td>
</tr>
<tr>
<td>Heat-aggregated FITC-IgG + monomeric IgG</td>
<td>28.46</td>
</tr>
</tbody>
</table>

*Five × 10^-7 M FITC-IgG was used to mark 1 × 10^6 bone marrow macrophages in the presence of PBS, or a 100-fold excess of either monomeric or heat-aggregated IgG. These data are from a representative experiment which has been repeated three times.

**Mean fluorescence channel.

TABLE 3. IgG Subclass Binding of FcR for Monomeric and Aggregated IgG on Bovine Bone Marrow Culture-Derived Macrophages

<table>
<thead>
<tr>
<th></th>
<th>Specific binding (MFC*)</th>
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<tbody>
<tr>
<td></td>
<td>Monomeric</td>
</tr>
<tr>
<td>IgG1</td>
<td>2.49</td>
</tr>
<tr>
<td>IgG2a</td>
<td>4.84</td>
</tr>
<tr>
<td>IgG2b</td>
<td>1.72</td>
</tr>
</tbody>
</table>

*One million bone marrow culture-derived macrophages were incubated with 1,000 u/ml of rBoIFNy for 24 or 48 h to examine expression of FcR for aggregated and monomeric subclasses of IgG, respectively. As will be substantiated later, higher mean fluorescence channels were afforded by rBoIFNy stimulation. The results are from a representative experiment which has been repeated three times. Specific binding represents the MFC of cells labelled with 5 × 10^-7 M of the subclass and fluorescein-conjugated F(ab')2 fragment of anti-IgG minus the MFC of cells additionally labelled in the presence of a 100-fold excess of unconjugated subclass.

**Mean fluorescence channel.

Fig. 2. Time course of rBoIFNy and rBoIFNα1 effects on macrophage expression of FcR for heat-aggregated bovine IgG. Treatment of cells was as described in Figure 1. Similar results were obtained in each of four trials.

blocked by heat-aggregated antibody and not by a similar 100-fold excess of monomeric IgG.

**FcR binding of immunoglobulin subclasses.** IgG2a proved to be the subclass of bovine immunoglobulin which predominately bound to the FcR for monomeric IgG (Table 3). A hierarchy of subclass binding to the
FcR for aggregated antibody was observed, where IgG1 > IgG2b > IgG2a. The level of nonspecific binding was equivalent for each subclass.

IFN Effects on Bovine FcR

Time course of FcR induction by either rBoIFNγ or rBoIFNα1. No significant increase in expression of FcR for either monomeric (Fig. 1) or heat-aggregated (Fig. 2) IgG could be observed with up to 6 h of exposure to 1,000 u/ml of either rBoIFNγ or rBoIFNα1. With 12 h of treatment with either IFN, enhancement of specific binding of fluorescent heat-aggregated IgG had begun, while binding of monomeric antibody was still not greatly increased. Induction of FcR for heat-aggregated IgG was maximal by 24 h of exposure to either of the IFNs, after which the response declined and had returned to starting levels by 48 h. In contrast, expression of FcR for monomeric IgG continued to increase through 24 to 48 h of IFN treatment. The extent of nonspecific binding was unaffected by either IFN. The different time course of induction of FcR for monomeric or aggregated IgG was not dependent on dose, since lower IFN concentrations produced temporally similar results, but less pronounced FcR up-regulation (data not shown).

Effect of IFN dose on FcR expression. Induction of FcR for heat-aggregated and monomeric IgG was examined at the optimal time of expression for each, 24 and 48 h, respectively. Specific binding to the FcR for both monomeric and aggregated IgG increased in a dose-dependent fashion from 1 to 1,000 u/ml of either rBoIFNγ or rBoIFNα1, the highest dose of each that was examined (Fig. 3). The potency of rBoIFNγ in enhancing expression of either type of FcR was approximately tenfold greater than that of rBoIFNα1, as can be seen by displacement of the α and γ plots from each other. In the unstimulated state, macrophage fluorescence attributable to specific binding of FITC-IgG was higher for aggregated antibody than for monomeric. Upon stimulation with 1,000 u/ml of rBoIFNγ, background MFC remained the same, yet a doubling of the MFC representing binding of aggregated IgG to macrophages and a more than threefold increase in MFC for monomeric IgG were observed. In addition to increases in MFC, the percentage of cells that expressed positive fluorescence was dramatically increased by rBoIFNγ treatment: 1,000 u/ml caused an increase from 9.3% to 29.8%, and from 33.8% to 63.0%, for binding of monomeric and aggregated IgG, respectively.

Mixtures of rBoIFNγ and rBoIFNα1. A suboptimal dose, i.e., a total of 1 u/ml, of rBoIFNγ and rBoIFNα1 either alone or in combination (0.5 u/ml of each) was applied to bone marrow macrophages. Poten-
extent to which they bound monomeric and aggregated immunoglobulins, in their susceptibility to trypsinization, and in their hierarchy of binding of IgG subclasses. Further evidence for at least two classes of FcR was obtained from cross-inhibition studies. Two additional findings, made for the first time in any species, were of particular importance: 1) the expression of both types of FcR could be differentially regulated by either rBoIFNγ or rBoIFNα1, and 2) mixtures of rBoIFNγ and α1 potentiated the induction of macrophage FcR expression, compared to the effects of either type of interferon used alone.

In both human beings and mice, distinct FcR for monomeric and aggregated IgG have been described [14,18]. The mouse macrophage FcR for monomeric immunoglobulin, which could be degraded by trypsin, preferentially bound IgG2a [9,18,20]. In contrast, the mouse macrophage FcR for aggregates had the capacity to bind aggregated immunoglobulin of all IgG subclasses, did not bind uncomplexed IgG, and was resistant to the effects of trypsin [4,9,18]. The profile for binding of IgG to bovine FcR, as we have described here, roughly paralleled that of the mouse. Characterization of human FcR present on myelomonocytic lines has revealed a similar dichotomy in the binding of monomeric and aggregated IgG, with the exception that IgG1 was the subclass preferentially bound as a monomer [8,14]. Demonstration of two distinct types of FcR in a third species, the bovine, suggests that the characteristic of differing classes of macrophage FcR may be broadly conserved among mammals.

Comprehensive analysis of the effects that rBoIFNs have on FcR expression demonstrated that up-regulation of the two FcR types occurs at different times. Previous studies using bovine macrophages showed generally increased FcR expression after they were treated with rBoIFNα1, as assessed by rosetting with erythrocytes that had been coated with unfractionated antibody [2]. Other earlier examinations of the effect that IFNs have on the induction of mouse FcR have been reported at single time points [19,23]. In these studies, the number and surface density of both FcRI and FcRII were increased. Additionally, IFNγ was found to be more efficacious in enhancing expression, compared to either IFNα or IFNβ [6]. While the data of Perussia et al. [14], obtained with human myelomonocytic cells, are consistent with greater effectiveness of rHuIFNγ, the effect was seen only with the FcR for monomeric IgG and rHuIFNα was unable to cause a change. Results obtained in the bovine system are comparable to those that have been described for the mouse.

Low doses (1 u/ml) of rBoIFNα1 and rBoIFNγ were found to be without demonstrable effect on FcR expression, yet mixtures of these IFNs at 0.5 u each caused
increased expression of both the FcR for monomeric and aggregated IgG. Synergism between suboptimal doses of α/β (type I) and γ (type II) interferons has been reported for the induction of other macrophage functions [3,7,13]: FcR expression can now be included in that list.

Following stimulation with rBoIFNs, there was an increase in both the number of FcR expressed per cell and the number of cells that displayed FcR. Therefore, while a subpopulation of bovine macrophages constitutively expressed FcR, additional FcR expression by previously FcR-negative macrophages could be effected by IFN treatment. The percentage of cells induced to express FcR for either aggregated or monomeric IgG suggested that at least in the stimulated state, some macrophages had the capacity to express both types of FcR.

There may be functional significance to the differential regulation of FcR types by IFNs that we have demonstrated here. Engagement of FcR can trigger secretion of prostaglandins, lysosomal enzymes, and reactive oxygen intermediates [reviewed in 1]. Further, in the mouse macrophage phagocytic and cytolytic activities appear to be mediated by antibodies of different classes and consequently, different FcR. Cytolysis could be inhibited by aggregated IgG2b myeloma protein, while phagocytosis was primarily inhibited by IgG2a regardless of its state of aggregation [21]. It would be expected that IFN-induced alterations in bovine FcR expression would subsequently affect these associated macrophage functions. Hence, regulation of expression of FcR types by IFNs may be the focus for alteration of a myriad of macrophage functions.

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