

Interferon β , a Cofactor in the Interferon γ Production Induced by Gram-negative Bacteria in Mice

By Yasunory Yaegashi, Peter Nielsen, Andreas Sing, Chris Galanos, and Marina A. Freudenberg

From the Max-Planck-Institut für Immunbiologie, D-79108 Freiburg, Germany

Summary

The interferon (IFN) γ production of splenocytes from closely related C57BL/10ScSn (Sn) and C57BL/10ScCr (Cr) mice was compared. Concanavalin A and CD3 monoclonal antibodies induced high levels of IFN- γ in both Sn and Cr splenocytes. By contrast, treatment with gram-negative bacteria induced IFN- γ only in Sn splenocytes; in Cr splenocytes, the IFN- γ response was heavily impaired. The IFN- γ induction by bacteria requires the cooperation of IFN- γ -producing cells with macrophages. Depletion of macrophages from Sn splenocytes resulted in the loss of ability to produce IFN- γ after bacterial stimulation. Reconstitution with new Sn macrophages restored the IFN- γ responsiveness, whereas reconstitution with Cr macrophages failed to do so. Normal function of IFN- γ -producing cells and a defective function of macrophages of Cr mice was demonstrated by evidence showing that whole or macrophage-depleted Cr splenocytes, when supplemented with Sn macrophages, acquire the ability to produce IFN- γ in response to bacteria. A similar effect was achieved by supplementing Cr splenocytes with supernatants of bacteria-stimulated Sn macrophages or with recombinant murine IFN- β or IFN- α . Preincubation of active macrophage supernatants with antibodies to IFN- β suppressed the helper activity for Cr splenocytes. Moreover, the bacteria-induced production of IFN- γ by Sn splenocytes could be inhibited by antibodies to murine IFN- β . The results provide evidence that IFN- β is an important cofactor of IFN- γ induction, which is not induced in Cr mice by gram-negative bacteria.

C57BL/10ScCr (Cr)¹ mice, in contrast to the closely related C57BL/10ScSn (Sn) mice, are highly resistant to all LPS effects (1–4). Coutinho and Meo (1) presented evidence that Cr mice are mutated in the same genetic locus as another LPS-resistant mouse strain, C3H/HeJ. The LPS resistance of C3H/HeJ mice has been studied in the past by several workers and found to be the result of a mutation of the LPS gene locus on chromosome 4 (4–6). The product of this locus has not been identified yet. We have shown that Cr mice exhibit a second defect. They differ from Sn and C3H/HeJ mice by their inability to exhibit an IFN- γ response after infection with *Salmonella typhimurium*, *Plasmodium chabaudi chabaudi*, or treatment with killed *Propionibacterium acnes* (3, 7). The impaired production of IFN- γ in treated Cr mice was accompanied by the absence of development of sensitization toward the lethal effects of bacterial endotoxin (LPS). In Sn and C3H/HeJ mice, sensitization parallels the production of IFN- γ after treatment with microorganisms. This observation led finally to the identification of IFN- γ

as a mediator of the LPS hypersensitivity induced by infection (7). It seems therefore that the production of IFN- γ in the course of infection plays a dual role: inducing a higher resistance toward the pathogen itself (for review see reference 8) and increasing susceptibility toward its toxic products, such as LPS.

In vitro analysis of the impaired production of IFN- γ by Cr splenocytes indicated that the defect may be restricted primarily to microbial stimuli (3). The IFN- γ response of the Cr splenocytes to the T cell mitogen Con A was present, although in comparative studies with three other mouse strains it was somewhat lower (3). We felt that a closer analysis of the impaired IFN- γ production in Cr mice might give more insight into the rather complicated mechanism of IFN- γ production. As known from previous studies, T and NK cells represent the main source of IFN- γ in the animal host (9–13). NK cells have been shown to exhibit rapid IFN- γ responses to bacteria and their products in vitro and in vivo and require the accessory help of macrophages (13–16; for a review see reference 8).

This study shows that Cr splenocytes exhibit impaired IFN- γ production after stimulation with all gram-negative bacteria so far tested. It is shown that IFN- γ -producing cells of Cr

¹ Abbreviations used in this paper: Cr, C57BL/10ScCr; ds, double-stranded; Sn, C57BL/10ScSn.

mice are normal, whereas the macrophages of these mice are unable to provide the required accessory help. Furthermore, evidence is presented that macrophage-produced IFN- β is an important cofactor in the induction of IFN- γ by gram-negative bacteria, and this is absent in bacteria-stimulated Cr cultures.

Materials and Methods

Animals. Sn, Cr, and BALB/c mouse strains were obtained from the breeding stock of the Max-Planck-Institut für Immunbiologie. 6–8 wk-old mice of either sex were used as tissue donors in all experiments.

Bacteria. *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Escherichia coli* J5, and *S. typhimurium* (C5) were obtained from overnight cultures. They were washed twice with PBS, pH 7.2, and killed by heating in PBS at 100°C for 30 min. All heat-killed bacteria were centrifuged, washed twice with distilled water, and lyophilized.

Antibodies. Two hybridoma cell lines producing rat mAb to mouse IFN- γ were kindly donated by G. L. Spitalny (R4-6A2; Trudeau Institute, Saranac Lake, NY) (17) and S. Landolfo (AN18.17.24; Institute of Microbiology, University of Torino, Torino, Italy) (18), respectively. mAbs were isolated from the hybridoma supernatants as described previously (7). Anti-CD3 mAb (19) was kindly provided by I. Müller (University of Lausanne, Lausanne, Switzerland). Polyclonal anti-IFN- β was prepared in a rabbit immunized four times with increasing amounts of a recombinant murine IFN- β (10–50 μ g) in IFA. The antiserum obtained was highly active (up to a dilution of 1:10,000) in immunoblot analysis with murine rIFN- β as antigen. The preimmune serum of the same rabbit served for control experiments. Monoclonal anti-IFN- β (rat IgG1; protein concentration 10 mg/ml) was purchased from Yamasa Shoyu (Tokyo, Japan). Monoclonal anti-IFN- α (rat IgG1; 200 μ g/ml) was purchased from GIBCO BRL (Gaithersburg, MD). Rat IgG (10 mg/ml), purified from normal rat serum as described earlier (7), was used in control experiments.

Chemicals. Recombinant murine IFN- γ was kindly provided by G. R. Adolph (Bender & Co., Vienna, Austria). Recombinant murine IFN- β was a kind gift from Dr. Moryama (Toray Industries Inc., Tokyo, Japan). Recombinant murine IFN- α was purchased from GIBCO BRL. Con A was purchased from Pharmacia (Freiburg, Germany). Double-stranded (ds) RNA [Poly(I) .Poly(C)] was purchased from Boehringer Mannheim (Mannheim, Germany). LPS of *Salmonella abortus equi* was prepared as described previously (20).

Cells. Macrophages were derived from bone marrow precursor cells flushed from mouse femora as previously described (21). Spleen cell suspensions were prepared at a concentration of 10^7 cells/ml of a high-glucose formulation of DME as previously described (22). The cells were cultured in plastic 96-well plates (Nunc, Roskilde, Denmark) in a volume of 200 μ l, or in 6-well plates (Costar, Cambridge, MA) in a volume of 3 ml in a humidified atmosphere containing 8% CO₂ at 37°C for 24 h. For determination of IFN- γ , culture supernatants were stored in aliquots at –80°C. Macrophage-depleted spleen cells were prepared as follows: Spleen cells were incubated at a concentration of 10^8 cells/ml in DME containing carbonyl-iron (reduced pentacarbonyl iron; Sigma, Deisenhofen, Germany) at a concentration of 200 mg/ 10^8 cells for 1 h at 37°C in a humidified atmosphere containing 8% CO₂. Phagocytes containing carbonyl-iron were removed from the spleen cell suspensions along with free carbonyl-iron by a magnet. The remaining cells were suspended in DME at a concentration of 10^7 cells/ml. FACS[®] analysis (Becton Dickinson & Co., Mountain View, CA) of spleen cells before and after this treatment indicated that the number of Mac-1⁺ (23) cells (macrophages) decreased from 5 to

<0.2% of the total cell population and that the percentage of 6B2⁺ (24) cells (B cells) increased from 59 to 67%. There were no significant changes in the concentrations of Thy-1⁺ (25) and NK1.1⁺ (26) cells (T and NK cells). The mAbs to Mac-1, 6B2, and Thy-1 were kindly provided by R. Carsetti (Max-Planck-Institut für Immunbiologie) and to NK1.1 by S. Vogel (Uniformed Services University of the Health Sciences, Bethesda, MD).

Measurement of IFN- γ . Concentrations of IFN- γ in culture supernatants were determined by a previously described specific ELISA technique (27). All IFN- γ values represent the average of four identical cultures; the SD was always <20%. The detection limit was 1–5 U IFN- γ /ml.

Northern Blot Analysis. Total RNA was isolated by a guanidinium isothiocyanate–phenol–chloroform/isoamylalcohol procedure (28). Briefly, 3×10^7 cells were suspended in 0.5 ml of solution D, which contained 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1 M 2-ME. Subsequently, 50 μ l of 2 M sodium acetate (pH 4), 0.5 ml of water-saturated phenol, and 100 μ l of chloroform/isoamylalcohol (25:1) were added. The mixture was incubated on ice for 15 min, and phase separation was accomplished by centrifugation at 4°C. Total RNA was precipitated from the aqueous phase by the addition of one volume of isopropanol after chilling to –20°C. The precipitates were collected by centrifugation, resuspended in 0.3 ml of solution D, and reprecipitated with 0.3 ml isopropanol. The RNA pellets were washed twice with 75% ethanol and resuspended in 10 μ l H₂O. The RNA concentration was determined by absorbance at 260 nm. RNA samples (3–5 μ g) were fractionated on 1% denaturing agarose–formaldehyde gels by use of standard conditions (29). The RNAs were transferred to Nytran filters (Schleicher & Schuell, Dassel, Germany) by capillary blotting and UV cross-linking to the filter. RNAs were hybridized overnight at 42°C with random-primed ³²P-labeled cDNA probes. The IFN- γ probe was a 700-bp cDNA fragment (EcoRI/BglII) of mouse IFN- γ provided by D. Stüber (Roth, Basel, Switzerland). IFN- β probe was a 571-bp PCR fragment spanning the complete open reading frame for murine IFN- β cloned into the SmaI site of pTZ19 (Pharmacia). The conditions for the amplification were 94°C for 40 s, 59°C for 30 s, and 72°C for 1 min with 300 ng mouse genomic DNA in a 50 μ l reaction volume, 200 μ M dNTP, 0.2 U Super Taq (Stehelin, Basel, Switzerland) and 0.5 μ M primers (5'-CCAGCCTGGCTTCCATCA-3' and 5'-GGTCTTCAGTTTGGGAAG-3'). After hybridization, the membranes were washed in 2 \times SSC for 10 min at room temperature, followed by 30 min at 42°C in 0.2 \times SSC containing 0.1% SDS. Autoradiography was performed by use of XOMATAR film (Eastman Kodak Co., Rochester, NY) at –80°C with an intensifier screen. The intensity of the ribosomal protein S12 mRNA (30), detected in different samples on the same blot, was used to control RNA loading on the Northern blot.

Results

Induction of IFN- γ in Sn and Cr Splenocytes by Con A, Anti-CD3 mAbs, and Killed Gram-negative Bacteria. Stimulation with optimal concentrations of either Con A (4 μ g) or anti-CD3 mAb (10 μ g) led to IFN- γ production in both Sn and Cr splenocyte cultures. Con A induced a somewhat lower response in Cr (on average 80 U/ml) than in Sn cultures (120 U/ml), whereas the response to anti-CD3 (115 versus 110 U/ml) was comparable in both cultures. Treatment with different gram-negative bacteria (*S. typhimurium*, *E. coli* J5, *Proteus mirabilis*, and *Pseudomonas aeruginosa*), however, in-

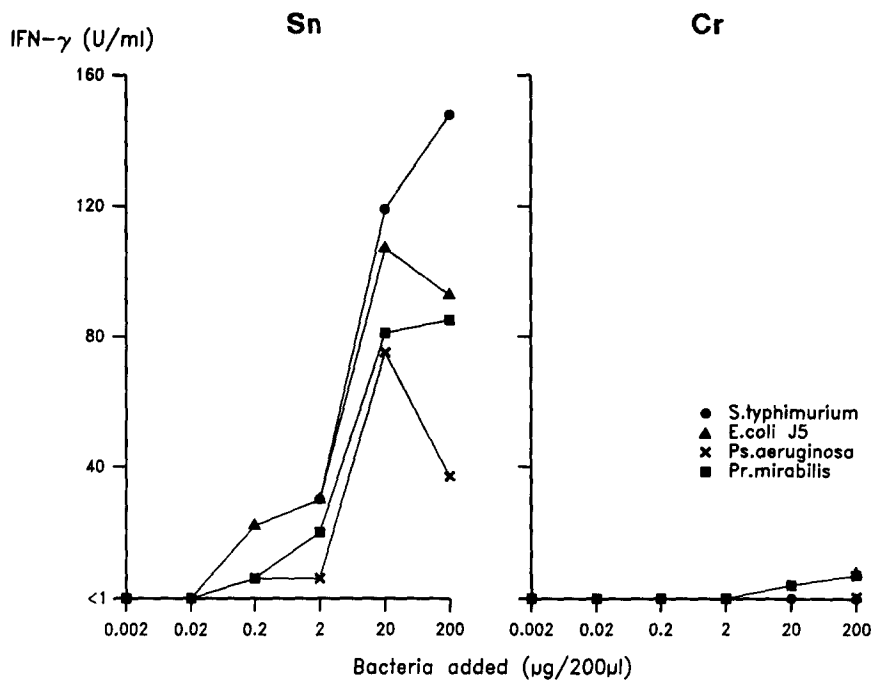


Figure 1. IFN- γ production by splenocytes after stimulation with different gram-negative bacteria. The IFN- γ response of Sn and Cr splenocytes (10^7 /ml) cultured with different amounts (0.02–200 μ g) of *S. typhimurium*, *E. coli* J5, *P. aeruginosa*, and *P. mirabilis* was measured as described in Materials and Methods. One of five representative experiments is shown.

duced IFN- γ only in Sn splenocytes, whereas in Cr cultures it was virtually ineffective (Fig. 1). Similar results were obtained with additional strains of *Salmonella* and *E. coli* (not shown). These results show that Cr mice, while capable of producing IFN- γ in response to Con A or anti-CD3 mAb, exhibit a heavily impaired response to gram-negative bacteria.

Kinetics of IFN- γ mRNA Induction and IFN- γ Production in Sn and Cr Splenocytes Stimulated by *S. typhimurium*. Fig. 2 shows cellular IFN- γ mRNA levels and IFN- γ concentrations in supernatants of Sn and Cr splenocytes at different times of *S. typhimurium* treatment. Northern blot analysis revealed a low level of IFN- γ mRNA in unstimulated cells of both mouse strains. After stimulation, IFN- γ mRNA and IFN- γ production increased temporarily in Sn splenocytes (maximum values at 8 and 12 h, respectively). In Cr splenocytes, neither mRNA increase nor IFN- γ production was observed.

Impaired Accessory Function of Cr Macrophages in the IFN- γ Response to Bacteria. Macrophages play an accessory role in the response of IFN- γ -producing cells to bacterial stimuli (8, 13–15, 31). We therefore investigated whether macrophages are required in the IFN- γ response to *S. typhimurium* and, if so, whether Cr macrophages are able to provide this function. Sn splenocytes were depleted of macrophages as described in Materials and Methods and their IFN- γ response to a standard dose of *S. typhimurium* (20μ g/ 2×10^6 cells) was measured. Fig. 3 shows that removal of macrophages abolished the ability of splenocytes to produce IFN- γ . Reconstitution with new, bone marrow-derived Sn macrophages restored the IFN- γ response. The amount of IFN- γ produced increased with higher numbers of macrophages added. The results confirm the essential role of macrophages in the IFN- γ response to bacteria. Reconstitution of Sn splenocytes with Cr

macrophages failed to restore IFN- γ response to *S. typhimurium* (Fig. 3), indicating an impaired accessory function of Cr macrophages.

The defect of Cr macrophages was confirmed in experi-

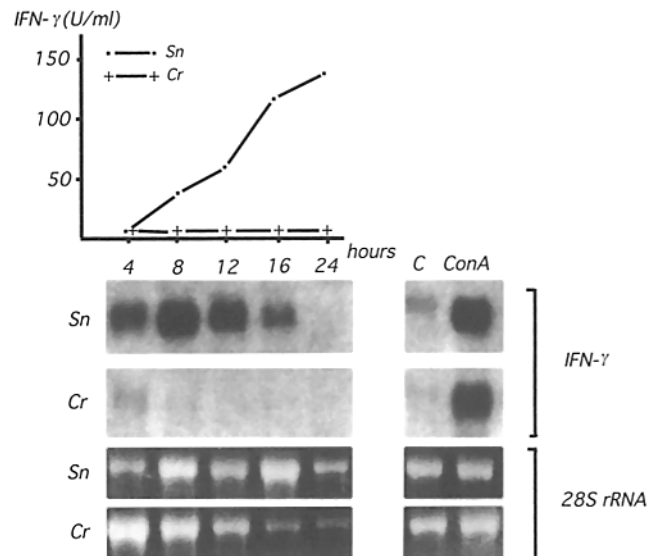


Figure 2. Time course of mRNA expression and secretion of IFN- γ by splenocytes stimulated with *S. typhimurium*. Splenocytes of Sn and Cr mice (3×10^7 cells/3 ml) were cultured for 0, 4, 8, 12, 16, and 24 h with *S. typhimurium* (300μ g). 16-h stimulation with Con A (30μ g) served as positive control. IFN- γ in culture supernatants was determined by an ELISA. IFN- γ mRNA was determined as a single band at 0.8 kb pair by Northern blot analysis as described in Materials and Methods. Amounts of total RNA applied to the electrophoresis gel for each sample is visualized by the intensity of the ethidium bromide-stained bands (28S and 18S ribosomal RNA). One of two representative experiments is shown.

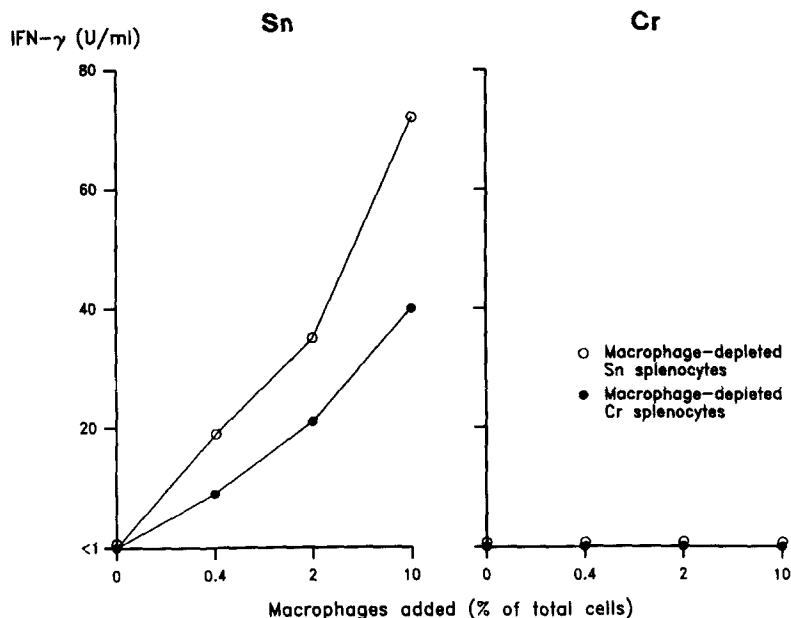


Figure 3. IFN- γ production by macrophage-depleted splenocytes reconstituted with Sn or Cr macrophages and stimulated with *S. typhimurium*. Macrophage-depleted Sn and Cr splenocytes and macrophages derived from bone marrow precursors were obtained as described in Materials and Methods. 2×10^6 macrophage-depleted splenocytes were reconstituted with the indicated numbers of new macrophages and cultured for 24 h with *S. typhimurium* (20 μ g) in a total volume of 200 μ l. IFN- γ in culture supernatants was determined by a specific ELISA. One of four representative experiments is shown.

ments in which whole or macrophage-depleted Cr splenocytes were supplemented with Sn or Cr macrophages and stimulated with bacteria. In such cultures, only addition of Sn macrophages enabled IFN- γ response. Addition of Cr macrophages was without effect (Figs. 3 and 4). Reconstitution of Cr splenocytes with macrophages of a nonrelated mouse strain (BALB/c) led also to IFN- γ production (not shown).

Soluble Factor(s) from Stimulated Sn Macrophages Repair(s) the IFN- γ Response of Cr Spleen Cells. It is known that soluble accessory factors are required for the induction of IFN- γ (15, 32, 33). The following experiments indicate that the induction of IFN- γ by gram-negative bacteria is supported by a macrophage factor that is not produced by Cr macrophages. Sn macrophages (10^6 /ml) were cultured in the presence of

S. typhimurium (100 μ g) for varying periods of time (1–24 h). Different amounts of the resulting culture supernatants (4–100 μ l) were added to Cr spleen cells (2×10^6) together with 20 μ g of the different bacteria, and IFN- γ was measured after 24 h of culture. Addition of the supernatant to Cr spleen cells conferred the ability to produce IFN- γ in response to all bacteria tested. The helper activity was first detected after 4 h of stimulation, reached its maximum at 8–12 h, and decreased thereafter (not shown). Fig. 5 illustrates the induction of IFN- γ in Cr splenocyte cultures supplemented with different amounts of 8-h supernatant. The macrophage supernatant alone, without bacteria, did not induce IFN- γ . IFN- γ was also absent in the controls containing bacteria and supernatants from unstimulated Sn macrophages cultured

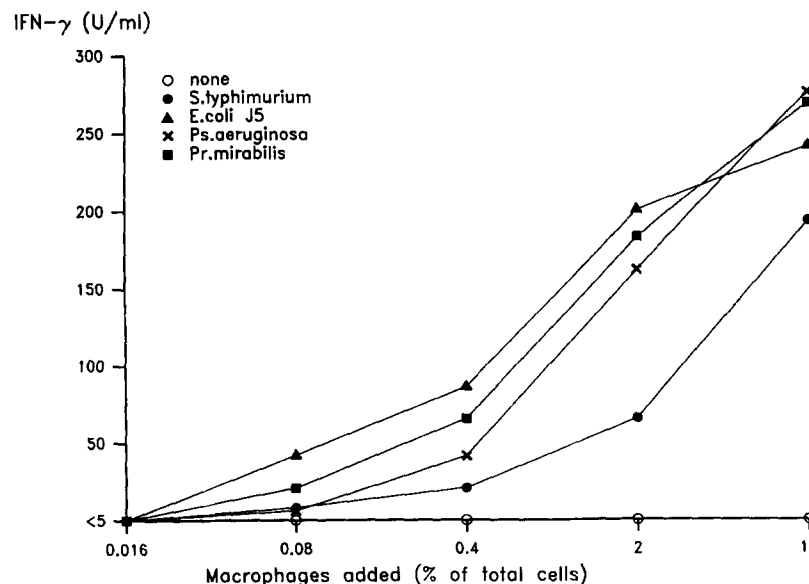


Figure 4. IFN- γ response of Cr splenocytes, supplemented with Sn macrophages, to bacteria. Splenocytes and macrophages were prepared as described in Materials and Methods. Different numbers of Sn macrophages were mixed with Cr splenocytes in the percentages indicated (final concentration 10^7 cells/ml) and stimulated with different bacteria (100 μ g/ml) for 24 h. IFN- γ in culture supernatants was determined by a specific ELISA. One of four representative experiments is shown.

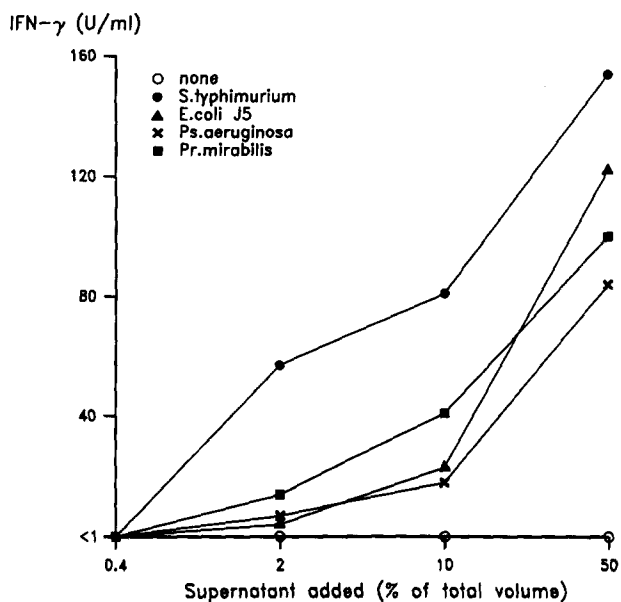


Figure 5. IFN- γ response of Cr splenocytes, supplemented with a supernatant of *S. typhimurium*-stimulated Sn macrophages, to bacteria. Splenocytes and macrophages were prepared as described in Materials and Methods. Cr splenocytes were supplemented with different amounts of supernatant obtained from Sn macrophages (10^6 /ml) stimulated for 8 h with *S. typhimurium* ($100 \mu\text{g}$). IFN- γ production by these splenocytes (10^7 /ml) stimulated with different bacteria ($100 \mu\text{g}$) was determined as described in Materials and Methods. One of three representative experiments is shown.

for different periods of time (4–24 h; not shown in Fig. 5). In similar experiments, supernatants from *S. typhimurium*-treated Cr macrophages did not support induction of IFN- γ by bacteria.

Other bacteria investigated (*E. coli*, *P. mirabilis*, *P. aeruginosa*; $100 \mu\text{g}/10^6$ cells) also induced the accessory factor in Sn macrophages. In addition, LPS ($0.1 \mu\text{g}$) and dsRNA ($10 \mu\text{g}$) were found to be potent inducers. In Cr macrophages, all bacteria and LPS were ineffective, whereas dsRNA induced the factor to the same level as in Sn macrophages. Thus, the inability of Cr macrophages to produce the factor involved in the induction of IFN- γ may be restricted to certain stimuli, such as gram-negative bacteria and their components.

The Macrophage Factor Required for IFN- γ Induction and Absent in Cr Cultures Is IFN- β . In searching for the helper factor absent in Cr macrophages, we examined the possibility of IFN- α and IFN- β being the relevant factors since these are known to be inducible by LPS and dsRNA (34–36). Other macrophage products known to be involved in the induction of IFN- γ (14, 15, 32, 33) could be excluded for reasons given in Discussion. Monoclonal antimurine IFN- α and IFN- β and a polyclonal anti-IFN- β were tested for their ability to suppress the helper activity present in supernatants from *S. typhimurium*-stimulated Sn macrophages. The supernatants were incubated with antibodies (1 – $50 \mu\text{l}/\text{ml}$) for 1 h at room temperature. $100 \mu\text{l}$ of the incubation mixture was then added to cultures of Cr spleen cells ($2 \times 10^6/100 \mu\text{l}$) and stimulated with *S. typhimurium* ($20 \mu\text{g}$). IFN- γ measurements showed a dose-dependent inhibition of helper activity (up

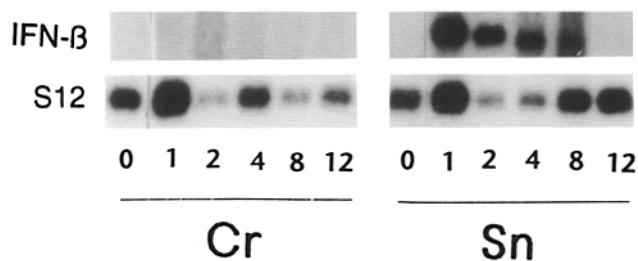


Figure 6. Expression of IFN- β mRNA of macrophages stimulated with *S. typhimurium*. Sn and Cr macrophages were prepared as described in Materials and Methods. 3×10^6 macrophages/3 ml were stimulated with *S. typhimurium* ($300 \mu\text{g}$) for the times indicated. Thereafter, total RNA was isolated, and IFN- β and ribosomal S12 mRNA were detected by Northern blot analysis, as described in Materials and Methods. One of two representative experiments is shown.

to 85%) by the monoclonal and polyclonal anti-IFN- β , whereas monoclonal anti-IFN- α and the control antibodies exhibited no suppressive effect (not shown).

Total RNA, obtained at different times of culture from *S. typhimurium*-stimulated Sn and Cr macrophages, was analyzed by Northern blot with an IFN- β -cDNA probe (Fig. 6). In unstimulated macrophages of either strain, IFN- β mRNA was not detectable. In Sn macrophages, strong induction of IFN- β mRNA was observed between 1 and 8 h of stimulation. In Cr macrophages, IFN- β mRNA was never detectable. Induction of IFN- β mRNA in Sn and its com-

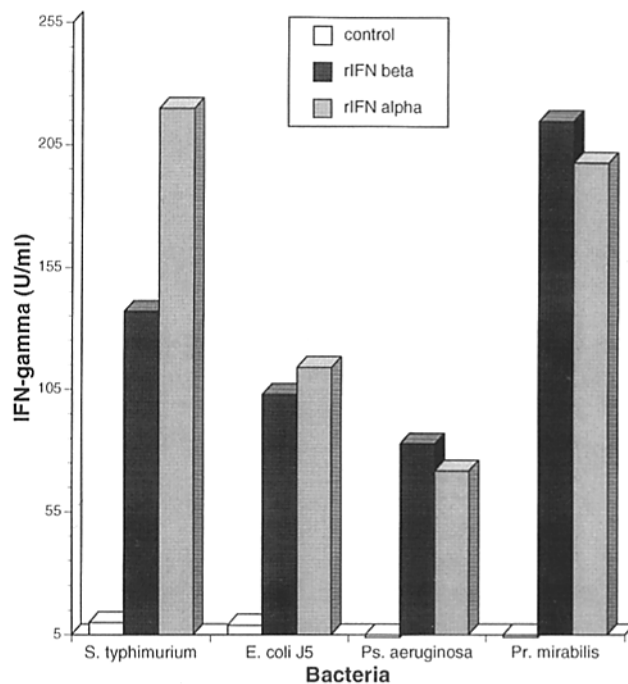


Figure 7. IFN- γ production by Cr splenocytes supplemented with rIFN- β or rIFN- α and stimulated with bacteria. IFN- γ response of splenocytes ($2 \times 10^6/200 \mu\text{l}$) supplemented with IFN- β or IFN- α ($10,000 \text{ U}$) and cultured with the different bacteria indicated ($20 \mu\text{g}$) was measured as described in Materials and Methods. One of four representative experiments is shown.

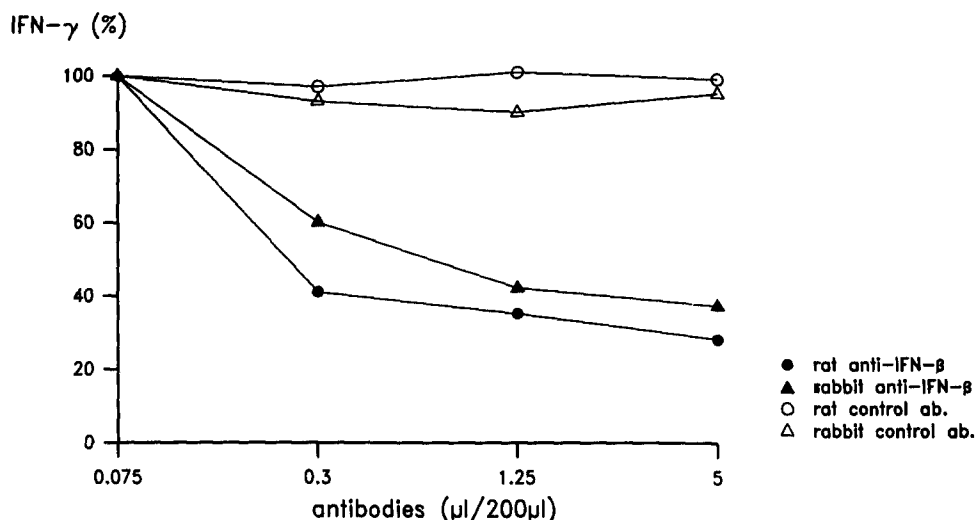


Figure 8. Inhibition of IFN- γ response to *S. typhimurium* in Sn splenocytes by anti-IFN- β . IFN- γ production by Sn splenocytes ($2 \times 10^6/200 \mu\text{l}$) supplemented with the indicated amounts of a monoclonal (10 mg protein/ml) or polyclonal anti-IFN- β and stimulated with *S. typhimurium* was measured as described in Materials and Methods. Preimmune rabbit serum and a rat IgG preparation served as controls. One of four representative experiments is shown.

plete absence in Cr macrophages were also seen after stimulation with LPS ($0.1 \mu\text{g}/10^6$ cells). Stimulation with dsRNA ($10 \mu\text{g}/10^6$ cells), however, induced a strong IFN- β message in both Sn and Cr macrophages (not shown).

We then investigated the effect of murine recombinant IFN- β on the defective IFN- γ response of Cr splenocytes. Addition of IFN- β ($5\text{--}50,000 \text{ U}/2 \times 10^6$ splenocytes) enabled, in a dose-dependent manner, the induction of IFN- γ by *S. typhimurium*. Fig. 7 shows that not only IFN- β but also recombinant IFN- α enabled a high production of IFN- γ in Cr splenocytes stimulated with different gram-negative bacteria.

IFN- β Antibodies Inhibit Induction of IFN- γ . The extent to which endogenous IFN- β is required for the induction of IFN- γ by gram-negative bacteria was investigated by use of antibodies against IFN- β . Both anti-IFN- β preparations inhibited the IFN- γ response of Sn splenocytes to *S. typhimurium* in a dose-dependent manner (Fig. 8). With the highest amount of anti-IFN- β ($5 \mu\text{l}/2 \times 10^6$ cells), $\sim 60\text{--}70\%$ inhibition was achieved. This amount of antibody inhibited the induction of IFN- γ (up to 80%) by all bacteria used in this study (not shown). Neither anti-IFN- α nor the control immunoglobulins exhibited an inhibitory effect. Thus, in mice, IFN- β is an important cofactor in the induction of IFN- γ by gram-negative bacteria.

Discussion

This present study demonstrates that splenocytes of Cr mice, in contrast to splenocytes of closely related Sn mice, exhibit defective IFN- γ production in response to gram-negative bacteria. This finding is in agreement with our previous results showing that IFN- γ production in Cr mice infected with different microorganisms is impaired (3, 7). Cr mice, however, do not possess a general defect in IFN- γ response, since Cr splenocytes produce high levels of IFN- γ after stimulation with Con A or anti-CD3.

The use of macrophage-depleted spleen cells from Sn and Cr mice and their reconstitution with macrophages of either

strain enabled an analysis of the IFN- γ defect in Cr mice. The absence of the IFN- γ response to *S. typhimurium* from macrophage-depleted Sn splenocytes and its restoration by addition of new Sn macrophages confirm the essential role of macrophages in the IFN- γ response. The inability of Cr macrophages to substitute for Sn macrophages shows that the former are defective. Consequently, when Cr splenocytes were supplemented with functionally intact Sn macrophages, they acquired the ability to produce IFN- γ after stimulation with all bacteria tested. This shows that in Cr mice, only the macrophages are defective, and the IFN- γ -producing cells themselves are normal.

Macrophages are believed to interact directly with IFN- γ -producing cells (31) and are known to secrete factors essential for IFN- γ induction (14, 32, 33). We show here that the defect of Cr macrophages is related to the absence of at least one soluble factor. Factors involved in IFN- γ induction and that may derive from macrophages are IL-1, TNF- α , and IL-12 (14, 15, 32, 33). Cr macrophages and splenocytes produce TNF- α and IL-1 (37; Freudenberg, M. A., and H. Loppnow, unpublished data) in response to bacteria. Regarding IL-12, we have detected induction of mRNA for both IL-12 subunits (38), preceding that of IFN- γ mRNA, in bacteria-stimulated Sn and Cr splenocytes (Yaegashi, Y., and M. A. Freudenberg, unpublished data). These data indicated that a macrophage product, not yet recognized as a cofactor of IFN- γ induction, is missing in Cr mice. The missing factor could be identified here as IFN- β for the following reasons. Expression of IFN- β mRNA in Sn macrophages correlated with the appearance of the factor in macrophage supernatants. Furthermore, exogenous IFN- β restored IFN- γ production in Cr splenocytes. Finally, IFN- β antibodies inhibited the helper effect of macrophage supernatants as well as the induction of IFN- γ in Sn splenocytes.

IFN- β shares the same cellular receptors with IFN- α , which explains why both cytokines exhibit similar biological activities (35). In this study, both recombinant IFNs (α and β) repaired the defect of Cr splenocytes. Thus both types of IFN may,

in principle, act as accessory factors in the production of IFN- γ . However, only anti-IFN- β and not anti-IFN- α inhibited the helper activity of macrophage supernatants and the induction of IFN- γ in stimulated Sn splenocytes. These data suggest that, in mice, bacteria induce predominantly IFN- β , as already shown for LPS (36).

The IFN- β defect of Cr mice, however, is not a general one since Cr macrophages are capable of producing IFN- β when stimulated with dsRNA. The reason why gram-negative bacteria do not induce IFN- β in Cr macrophages is not known. The most obvious explanation would be that LPS is the only IFN- β -inducing bacterial component and consequently cannot act in LPS-resistant Cr mice. In this case, the inability of Cr mice to produce IFN- β and subsequently IFN- γ would be directly related to their LPS nonresponsiveness. Gram-negative

bacteria, however, have been shown to induce a number of macrophage cytokines in Cr mice due to other non-LPS components that they contain. Thus, production of TNF- α (37), IL-1 and IL-6 (Loppnow, H., and M. A. Freudenberg, unpublished data) and GM-CSF (Kumazawa, Y., R. Gerle, and M. A. Freudenberg, unpublished data) were detected after stimulation with *S. typhimurium* and other gram-negative bacteria in Cr and C3H/HeJ mice, despite their LPS nonresponsiveness. These components, although obviously incapable of inducing IFN- β in Cr mice, may well do so in other mouse strains, including C3H/HeJ. This would explain the high levels of IFN- γ found in C3H/HeJ mice after stimulation with *S. typhimurium* (3). Further experiments are required to understand the defect of Cr mice and the complex mechanisms involved in IFN- γ induction.

We appreciate the excellent technical assistance of H. Stübig, C. Steidle, and N. Goos. We would like to thank R. Carsetti for help with and introduction to the FACS[®] analysis.

This study was supported in part by the Dr. Mildred Scheel Foundation for Cancer Research.

Address correspondence to Marina A. Freudenberg, Max-Planck Institut für Immunbiologie, Stübeweg 51, D-79108 Freiburg, Germany.

Received for publication 26 August 1994 and in revised form 2 November 1994.

References

1. Coutinho, A., and T. Meo. 1978. Genetic basis for unresponsiveness to lipopolysaccharide in C57BL/10 Cr mice. *Immunogenetics*. 7:17-24.
2. McAdam, K.P.W.J., and J.L. Ryan. 1978. C57BL/10/CR mice: nonresponders to activation by the lipid A moiety of bacterial lipopolysaccharide. *J. Immunol.* 120:249-253.
3. Freudenberg, M.A., Y. Kumazawa, S. Meding, J. Langhorne, and C. Galanos. 1991. Gamma interferon production in endotoxin-responder and -nonresponder mice during infection. *Infect. Immun.* 59:3484-3491.
4. Vogel, S.N. 1992. The Lps gene: Insights into the genetic and molecular basis of LPS responsiveness and macrophage differentiation. In *Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine*. B. Beutler, editor. Raven Press, New York. 485-513.
5. Watson, J., and R. Riblet. 1974. Genetic control of responses to bacterial lipopolysaccharides in mice. I. Evidence for a single gene that influences mitogenic and immunogenic response to lipopolysaccharides. *J. Exp. Med.* 140:1147-1161.
6. Watson, J., K. Kelly, M. Largen, and B.A. Taylor. 1978. The genetic mapping of a defective LPS response gene in C3H/HeJ mice. *J. Immunol.* 120:422-424.
7. Katschinski, T., C. Galanos, A. Coumbos, and M.A. Freudenberg. 1992. Gamma interferon mediates *Propionibacterium acnes*-induced hypersensitivity to lipopolysaccharide in mice. *Infect. Immun.* 60:1994-2001.
8. Sheehan, K.C.F., and R.D. Schreiber. 1992. The synergy and antagonism of interferon- γ and TNF. In *Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine*. B. Beutler, editor, Raven Press, New York. 145-178.
9. Marcucci, F., M. Waller, H. Kirchner, and P. Kramer. 1981. Production of immune interferon (IFN- γ) by murine T cell clones from long-term cultures. *Nature (Lond.)*. 291:79-81.
10. Nathan, I., J.E. Groopman, S.G. Quan, N. Bersch, and D.W. Glode. 1981. Immune (γ) interferon produced by a human T-lymphoblast cell line. *Nature (Lond.)*. 292:842-844.
11. Djeu, J.Y., N. Stocks, K. Zoon, G.J. Stanton, T. Timonen, and R.B. Herberman. 1982. Positive self regulation of cytotoxicity in human natural killer cells by production of interferon upon exposure to influenza and herpes viruses. *J. Exp. Med.* 156:1222-1234.
12. Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai. 1983. Natural killer (NK) cells as a responder to interleukin 2 (IL-2). II. IL-2 induced interferon- γ production. *J. Immunol.* 130:988-992.
13. Bancroft, G.J., R.D. Schreiber, G.C. Bosma, M.J. Bosma, and E.R. Unanue. 1987. A T cell-independent mechanism of macrophage activation by interferon-gamma. *J. Immunol.* 139:1104-1107.
14. Bancroft, G.J., K. C. Sheehan, R.D. Schreiber, and E.R. Unanue. 1989. Tumor necrosis factor is involved in T cell-independent pathway of macrophage activation in *scid* mice. *J. Immunol.* 143:127-130.
15. Wherry, J.C., R.D. Schreiber, and E.R. Unanue. 1991. Regulation of gamma interferon production by natural killer cells in *scid* mice: roles of tumor necrosis factor and bacterial stimuli. *Infect. Immun.* 59:1709-1715.
16. Ramarathinam, L., D.W. Niesel, and G.R. Klimpel. 1993. *Salmonella typhimurium* induces IFN-gamma production in murine splenocytes. Role of natural killer cells and macrophages. *J. Immunol.* 150:3973-3981.
17. Spitalny, G.L., and E.A. Havell. 1984. Monoclonal antibody

- to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. *J. Exp. Med.* 159:1560-1565.
18. Prat, M., G. Gribaudo, P.M. Comoglio, G. Cavali, and S. Landolfo. 1984. Monoclonal antibodies against murine γ -interferon. *Proc. Natl. Acad. Sci. USA.* 81:4515-4519.
 19. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374-1378.
 20. Galanos, C., O. Lüderitz, and O. Westphal. 1979. Preparation and properties of a standardized lipopolysaccharide from *S. abortus equi* (Novo-Pyrexal). *Zentralbl. Bakteriol. Parasitenk. D Infektionskr. Hyg. Abt. 1 Orig. Reihe A* 243:226-244.
 21. Freudenberg, M.A., D. Keppler, and C. Galanos. 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. *Infect. Immun.* 51:891-895.
 22. Tanamoto, K., C. Galanos, O. Lüderitz, S. Kusumoto, and T. Shiba. 1984. Mitogenic activities of synthetic lipid A analogs and suppression of mitogenicity of lipid A. *Infect. Immun.* 44:427-433.
 23. Van Voorhis, W.C., M.D. Witmer, and R.M. Steinman. 1982. The phenotype of dendritic cells and macrophages. *Fed. Proc.* 42:3114-3118.
 24. Ballas, Z.K., and W. Rasmussen. 1993. Lymphokine-activated killer cells. VII. IL-4 induces NK1.1⁺ CD8 α ⁺ β ⁻ TCR- $\alpha\beta$ B220⁺ lymphokine-activated killer subset. *J. Immunol.* 150:17-30.
 25. Reif, A.E., and J.M.V. Allen. 1963. Specificity of isoantisera against leukaemic and thymic lymphocytes. *Nature (Lond.)* 200:1332-1333.
 26. Sentman, C.L., J. Hackett, Jr., T.A. Moore, M.M. Tütt, M. Bennett, and V. Kumar. 1989. Pan natural killer cell monoclonal antibodies and their relationship to the NK1.1 antigen. *Hybridoma.* 8:605-610.
 27. Slade, S.J., and J. Langhorne. 1988. Production of interferon-gamma during infection with *Plasmodium chabaudi chabaudi*. *Immunobiology.* 179:353-365.
 28. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
 29. Wahl, G.M., J.L. Meinkoth, and A.R. Kimmel. 1988. Northern and Southern blots. In *Guide to Molecular Cloning Techniques*. S.L. Berger and A.R. Kimmel, editors. Academic Press, Orlando, FL. 572-581.
 30. Ayane, M., P. Nielsen, and G. Köhler. 1989. Cloning and sequencing of mouse ribosomal protein S12 cDNA. *Nucleic Acids Res.* 17:6722.
 31. Matsumura, H., and M. Nakano. 1988. Endotoxin-induced interferon-gamma production in culture cells derived from BCG-infected C3H/HeJ mice. *J. Immunol.* 140:494-500.
 32. Tripp, C.S., S.F. Wolf, and E.R. Unanue. 1993. Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA.* 90:3725-3729.
 33. Le, J., J.X. Lin, D. Henriksen-DeStefano, and J. Vilcek. 1986. Bacterial lipopolysaccharide-induced interferon-gamma production: roles of interleukin 1 and interleukin 2. *J. Immunol.* 136:4525-4530.
 34. Lengyel, P. 1982. Biochemistry of interferons and their action. *Annu. Rev. Biochem.* 51:251-282.
 35. Pestka, S.J., J.A. Langer, K.Z. Zoon, and C.E. Samuel. 1987. Interferons and their action. *Annu. Rev. Biochem.* 56:727-777.
 36. Vogel, S.N. 1992. Lipopolysaccharide-induced interferon. In *Bacterial Endotoxic Lipopolysaccharides*. Vol. II. Immunopharmacology and Pathophysiology. J.L. Ryan and D.C. Morrison, editors. CRC Press, Boca Raton, FL. 166-195.
 37. Freudenberg, M.A., and C. Galanos. 1991. Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-galactosamine-treated mice. *Infect. Immun.* 59:2110-2115.
 38. Gubler, U., A.O. Chua, D.S. Schoenhaut, C.M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A.G. Wolitzky, P.M. Quinn, P.C. Familletti, et al. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA.* 88:4143-4147.