Cancer Immunol Immunother (1989) 28: 107–115

Cancer Immunology Immunotherapy

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Endogeneous interferon α/β produced by murine Kupffer cells augments liver-associated natural killing activity*

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Summary. Nonparenchymal liver cells from untreated C3HeB/FeJ mice, when incubated in medium containing 10% fetal bovine serum or portal serum, produced significant amounts of interferon alpha/beta (IFNα/β). In contrast, other cell populations (spleen, mononuclear blood cells and peritoneal cells) from C3HeB/FeJ mice or nonparenchymal liver cells from other strains of mice (C3H/HeJ, germ-free C3H/HeN and C57Bl/6J) produced little or no detectable IFN in fetal bovine serum under the same culture conditions. The cells in the nonparenchymal liver cell population responsible for IFNα/β production were adherent, phagocytic, silica-sensitive, carbonyl-iron-sensitive, and Thy1.2$^-$, presumably Kupffer cells or resident liver macrophages. IFNα/β production by cultured Kupffer cells was not observed if medium containing fetal bovine serum or portal serum was treated with polymyxin B or if Kupffer cells were cultured in serum-free medium. This suggested that small amounts of endotoxin in fetal bovine or portal serum stimulated Kupffer cells to produce IFNα/β. Possibly, Kupffer cells are in a different state of activation/maturation than peritoneal and splenic macrophages since the sensitivity of resident Kupffer cells from C3HeB/FeJ mice to the stimulatory effects of endotoxin. The endogenous production of IFNα/β by Kupffer cells from C3HeB/FeJ mice can augment liver-associated natural killer (NK) activity against YAC-1 cells (4 h) and induce liver-associated cytotoxic activity, not restricted by the major histocompatibility complex, against NK resistant P815 mastocytoma cells (18 h).

Introduction

Nonparenchymal liver cells (NPC), which form the lining of the liver sinusoids [41], comprise a heterogeneous cell population, consisting mainly of endothelial cells, pit cells or large granular lymphocytes [17, 24] and Kupffer cells or resident liver macrophages [20]. Kupffer cells play an important protective role in the body by removing bacteria, viruses and tumors [25, 28, 29]. Endotoxin, the lipopolysaccharide moiety derived from the cell walls of enteric gram-negative bacteria, is constantly released in the gut upon death of the bacteria and transported via the portal venous circulation to the liver [14, 28, 30]. Kupffer cells are the principal cells in the liver that clear endotoxin from the portal blood [25, 29]. Endotoxin can induce a wide range of immunological and toxic phenomena in vivo, including fever, hypoglycemia, induction of acute-phase proteins and even death [26, 29]. In addition, endotoxin can induce the release of interferon (IFN) from peritoneal and bone-marrow-derived macrophages in vitro [9, 11].

Natural killer (NK) cells and cytotoxic effector cells, not restricted by the major histocompatibility complex (MHC), have lytic activity in vitro and in vivo against tumor cells and virally infected cells without prior sensitization of the host [12, 21, 40]. IFNα/β can further enhance NK-mediated lysis [33]. Since the liver is often a target of metastatic tumor cell invasion and viral infection, the interplay between Kupffer cells and its mediators with liver-associated NK cells or non-MHC-restricted cytotoxic effector cells may be crucial in the liver's host defense mechanism. Previously, we reported that Kupffer cells could maintain the lytic capacity of liver-associated natural killing in culture [2, 3]. The mechanism of this phenomenon was, therefore, investigated.

We present evidence that isolated murine Kupffer cells from C3HeB/FeJ mice produce IFNα/β in vitro in response to low levels of endotoxin normally present in fetal bovine serum or portal serum. This production of IFNα/β by Kupffer cells from C3HeB/FeJ mice can augment liver-associated NK activity against YAC-1 cells and induce liver-associated non-MHC-restricted cytotoxic activity against NK-resistant P815 mastocytoma cells.

Materials and methods

Mice. C57Bl/6, C3HeB/FeJ and C3H/HeJ mice (used at 6–12 weeks of age) were purchased from Jackson Laboratories, Bar Harbor, Me. Germ-free C3H/HeN mice were a gift from Dr. Pat Bealmear, Roswell Park Memorial Institute, Buffalo, NY.

* This work was supported by National Institutes of Health grant CA28835, VA Merit Grant and by the Margaret Duffy and Robert Cameron Troup Fund

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Abbreviations used: NPC, nonparenchymal liver cells; FBS, fetal bovine serum; IFN, interferon; α-AsGm-1, anti-asialo-GM1; α-Thy1.2, anti-Thy1.2; Hepes, 4-(2-hydroxyethyl)1-piperazine-ethanesulfonic acid; HBSS, Hanks' balanced salt solution; GBSS, Gey's balanced salt solution; SRBC, sheep red blood cells; Ab, mouse anti-SRBC; NK, natural killer; MHC, major histocompatibility complex; polyI · polyC, polyinosinec · polycytidylic acid

Medium. Tissue-culture medium consisted of RPMI 1640 medium supplemented with 25 mM Hepes, gentamicin (50 μg/ml) and 10% FBS (GIBCO, Grand Island, NY).

Reagents. Lipopolysaccharide (*Escherichia coli* 0127:B8) was purchased from Difco Laboratories (Detroit, Mich) polyI·polyC, polymyxin B and collagenase type IV were purchased from Sigma Chemical Co. (St. Louis, Mo). Bovine serum albumin was purchased from Calbiochem (San Diego, Calif). Murine IFNα/β was purchased from Lee Biomolecular (San Diego, Calif) and murine IFNγ was a gift from Dr. Edward Havell, Trudeau Institute.

Preparation of NPC. NPC were isolated by modifying a method already described elsewhere [3, 31]. Briefly, mice were sacrificed by cervical dislocation, the skin over the abdomen and chest was reflected, and the inferior vena cava was ligated above the diaphragm. The inferior vena cava below the liver was cannulated with a heparinized 20-gauge needle, through which the liver was perfused at 37° C with 40 ml HBSS without Ca^{2+} and Mg^{2+} (pH 7.2), delivered over 2 min with the outflow through an incision in the portal vein. The liver was then perfused with 100 ml sterile HBSS (pH 7.6) supplemented with 0.05% collagenase, delivered over 5 min. The distended and blanched liver was removed, rinsed with HBSS, teased apart with a rubber policeman and passed through a 100-μm nylon mesh screen. A single-liver-cell suspension (5 ml) was mixed with 7 ml 30% w/v Metrizamide (Accurate and Scientific Co., Hicksville, NY), dissolved in GBSS (without NaCl), then gently overlaid with 1 ml GBSS (with NaCl) and centrifuged at 550 g for 15 min at 23° C. NPC were collected from the interface, washed twice in HBSS and resuspended in tissue-culture medium. NPC obtained by this method were >95% viable (as assessed by trypan blue exclusion) and contained less than 3% hepatocytes. Approximately 10×10^6 NPC were recovered per liver.

Preparation of resident peritoneal exudate cells. Peritoneal exudate cells were harvested by lavage of the peritoneal cavity with 6 ml Ca^{2+}- and Mg^{2+}-free HBSS and aspiration of the fluid with a syringe through an 18-gauge needle.

Preparation of cells from the spleen or peripheral blood. Spleens were pushed through stainless-steel screens and the resulting cell suspension was filtered through nylon mesh. Cells were washed twice with HBSS and resuspended in tissue-culture medium. Blood was collected from mice by cardiac puncture and mixed with HBSS containing heparin (100 units/ml). Mononuclear blood cells were isolated by centrifugation at 400 g for 40 min at 23° C on a Ficoll-Hypaque gradient (Litton Bionetics, Kensington, Md).

Serology. The following antibodies were used in this study. Monoclonal anti-mouse Thy1.2 was purchased from New England Nuclear (Boston, Mass) and used at a final dilution of 1:300. Rabbit anti-asialo-GM1 was purchased from Wako Chemical Co. (Dallas, Tex) and used at a final dilution of 1:40. To eliminate NPC subpopulations, 20×10^6 NPC were trated with 0.1 ml antibody for 30 min at 4° C. The cells were centrifuged and resuspended in a 1:40 dilution of baby rabbit complement (Pel Freeze, Rogers, Ariz) for an additional 60 min at 37° C, washed twice in tissue-culture medium and assayed.

Isolation of cells not adherent to nylon wool. NPC were passed through nylon-wool columns according to an established procedure [16]. Briefly, NPC (4×10^7) were applied to the column and incubated for 60 min at 37° C before nonadherent cells were displaced by dropwise addition of 50 ml warm tissue-culture medium.

Isolation of cells adherent to plastic. Various numbers of NPC were seeded into microwells of 96-well tissue-culture plates and allowed to adhere for 3 h at 37° C in tissue-culture medium containing 20% FBS. After incubation, the nonadherent cells were removed and the plates washed four times with warm medium. More than 90% of adherent cells had the morphology of macrophages, as shown by uptake of latex beads.

Elimination of phagocytic cells. Silica, with an average particle size of 5 μm (Steinkohlenbergbauverein, Essen, FRG), was sterilized and sonicated before being resuspended in tissue-culture medium and added to cells at a final concentration of 1 mg/ml. Cells (1×10^7) were suspended in a total volume of 2 ml tissue-culture medium in 35-mm tissue-culture plates with 100 mg sonicated carbonyl iron powder (GAF Corporation, New York, NY) for 2 h at 37° C. The iron particles were drawn to one side of the plate with a magnet and the cells that did not ingest the iron particles were removed, washed twice in tissue-culture medium and assayed [22].

Assay of cell-mediated cytotoxic activity. The target cells were adapted to continuous growth in tissue-culture medium. Target cells were labeled with $Na_2{}^{51}CrO_4$ for 1 h at 37° C (100 μCi/10^6 cells). The ^{51}Cr-release assay was performed in 96-well flat-bottomed tissue-culture plates (Costar, Cambridge, Mass). NPC were mixed with 2×10^4 tumor target cells in triplicate in a total volume of 200 μl and incubated for 4–18 h in a humidified atmosphere of 5% CO_2 in air. Supernatants were harvested and counted in a Packard gamma counter. The results were expressed as the percentage specific release calculated by using the equation, $100(A-B)/(C-B)$, in which A = radioactivity of experimental, B = radioactivity from spontaneous release, and C = maximal radioactivity from target cells. Target cells used in this study were YAC-1 and P815 maintained in tissue-culture medium and free of mycoplasma.

Assay of phagocytic activity. The phagocytic activity of NPC was assessed as described by Cohen et al. [4]. Briefly, SRBC were labeled with ^{51}Cr and then coated with a 1:100 dilution of mouse anti-SRBC antiserum (Ab). NPC (or control unlabeled SRBC) were incubated with coated Ab^{51}Cr-SRBC for 4 h at 37° C. Nonphagocytized Ab ^{51}Cr-SRBC were then lysed for 15 s by the addition of distilled water followed by 2× concentrated HBSS. The cells were pelleted by centrifugation and the radioactivity in the pellet and in the supernatant (sup) was determined with a Packard gamma counter. The percentage phagocytic activity was calculated as given below (^{51}Cr measured as cpm):

$$\text{phagocytic activity (\%)} = \left[\frac{^{51}\text{Cr pellet}}{^{51}\text{Cr sup} + {}^{51}\text{Cr pellet}} (\text{exp}) - \right.$$

$$\left. - \frac{^{51}\text{Cr pellet}}{^{51}\text{Cr sup} + {}^{51}\text{Cr pellet}} (\text{control}) \right] \times 100$$

Each sample was assessed in triplicate and the values were expressed as the mean.

Limulus amoebocyte lysate assay. The concentration of endotoxin in FBS or albumin was determined by a *Limulus* amoebocyte lysate assay kit (QL1000) obtained from MA Bioproducts (Walkersville, Md).

Assay of murine IFN activity. IFN samples were prepared by incubation of cells in 96-well flat-bottomed tissue-culture plates for varying periods of time. IFN in cell-free supernatants was measured by inhibition of cytopathic effect of vesicular stomatitis virus against a mouse fibroblast L-cell line (L929) using the method of Finter [8]. Briefly, L cells were seeded into 24-cell tissue-culture plates (6×10^4 cells/well) and allowed to form confluent monolayers. The cells were then overlayed with serial dilutions of the test supernatant or reference IFN and incubated overnight at 37° C. After removal of the IFN, the cells were challenged with vesicular stomatitis virus (approx. 40 plaque-forming units per well). After 24 h, the cells were stained with a 1% solution of crystal violet and the percentage lysis determined. One unit of IFN was defined as the highest dilution causing a 50% reduction in cytopathic effect and is reported in NIH reference units per milliliter. The lower limit of detection in this assay was 20 units/ml.

Rabbit anti-mouse IFNα/β and rabbit anti-mouse IFNγ were purchased from Enzo Biochemical (New York, NY). A 1:280 dilution of anti-IFNα/β neutralized 500 units/ml IFNα/β while a 1:50 dilution of anti-IFNγ neutralized 500 units IFNγ. In order to determine the type of IFN present in the test supernatants, the appropriate anti-IFN was added to either the cells and incubated for 18 h or added directly to the supernatant and the residual IFN activity in each test supernatant was assayed as described above. Neutralization of antiviral activity by the specific antisera was not affected by dilution of the test supernatants during the IFN assay.

Results

IFN production by various cell populations from C3HeB/FeJ and C57Bl/6 mice

Since the liver is constantly exposed to gut-derived substances (i.e. endotoxin, bacteria, viruses [14, 28, 30], which induce IFN [9, 11], the ability of NPC from C3HeB/FeJ mice to produce IFN was determined. C3HeB/FeJ mice were used because they are known to be highly resistant to viral hepatitis [36]. NPC were cultured in 10% FBS at various cell densities and times and IFN activity assayed. As shown in Fig. 1, NPC produced optimal anti-viral IFN activity when plated at 4×10^5 cells/well. Furthermore, NPC produced peak anti-viral IFN activity after 18 h of culture (Fig. 2). NPC were compared with other cell populations from C3HeB/FeJ mice for anti-viral IFN activity. As shown in Table 1, NPC produced significant anti-viral IFN activity (mean: 220 units/ml ±16). In contrast, no

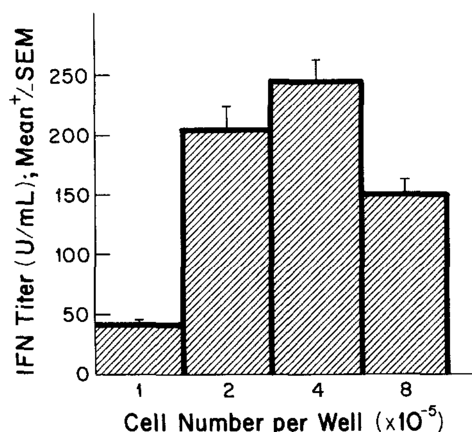

Fig. 1. Effect of cell concentration on IFN production. Results are expressed as mean ± SEM of 13 experiments (for 8×10^5), 15 experiments (for 4×10^5), 7 experiments (for 2×10^5) and 2 experiments (for 1×10^5)

detectable IFN activity (less than 20 units/ml) was found in cell-free culture supernatants from resident peritoneal exudate cells, spleen cells and mononuclear blood cells cultured under the same conditions as NPC. Serum from C3HeB/FeJ mice also displayed no detectable IFN activity. IFN produced by NPC was independent of the procedure used for their isolation since resident peritoneal exudate cells, treated with collagenase/Metrizamide, did not produce IFN. Interestingly, NPC from polyI-polyC-treated mice, did not produce IFN activity. In contrast, high levels of IFN activity were detected in the serum of these mice injected with the IFN inducer. NPC from C57Bl/6 mice, which are susceptible to viral hepatitis infection [36], produced only marginal levels of IFN activity (Table 1).

Characterization of the IFN-producing cell within the NPC population

To determine which cell population within NPC from C3HeB/FeJ mice was responsible for IFN production, several enrichment or depletion experiments were performed. Additionally, Fc-dependent phagocytic activity of the cell populations was assayed. Treatment of NPC with silica

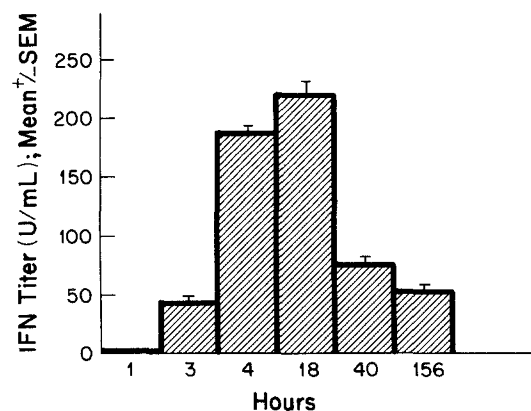

Fig. 2. Kinetics of IFN production. Supernatants from 4×10^5 NPC were collected at various time points and assayed for IFN activity. Results are expressed as mean ± SEM from 3 experiments

Table 1. IFN production by various cell populations from C3HeB/FeJ and C57B1/6J mice[a]

Strain	Cells/serum	IFN (units/ml)
C3HeB/FeJ	NPC	220±16[c] (range: 32–1034)
	Poly I · poly C (−4 h) NPC	50
	Poly I · poly C (−24 h) NPC	<20
	Resident PEC	<20
	Collagenase/Metrizamide resident PEC[b]	<20
	Spleen cells	<20
	Mononuclear blood cells	<20
	Normal serum	<20
	Poly I · poly C (−24 h) serum	1038
C57B1/6J	NPC	30±5 (range <20–59)[d]

[a] 4×10^5 NPC, 10^6 peritoneal exudate cells or [PEC] 10^6 spleen cells from untreated or poly I · poly C-treated mice (200 μg, i.p.) were cultured in RPMI 1640 medium containing 10% FBS for 18 h and their supernatants assayed for IFN activity
[b] PEC were incubated in 0.05% collagenase for 15 min at 37°C, washed three times in HBSS, layered over a Metrizamide gradient; the cells at the interface were collected and were cultured in RPMI 1640 medium containing 10% FBS for 18 h and their supernatants assayed for IFN activity
[c] Mean of 22 experiments ± SEM
[d] Mean of 3 experiments ± SEM

Table 2. Characterization of NPC producing IFN from C3HeB/FeJ mice

Expt	Treatment of NPC	IFN (units/ml)	Phagocytic activity[f]
A	None	225	16
	Silica[a]	<20	0
B	None	175	26
	Carbonyl iron[b]	<20	2
C	None	181	NT
	AD (plastic)[c]	162	NT
	NA (nylon)[d]	<20	NT
D	None	293	NT
	C	250	NT
	α-Thy1.2 antibody + C[e]	278	NT

[a] 4×10^5 NPC were incubated in 10% FBS with silica (1 mg/ml) for 18 h and supernatants were assayed for IFN activity
[b] 10×10^6 NPC were treated with 100 mg carbonyl iron for 1 h at 37°C and a strong magnet was used to remove phagocytic cells. The remaining cells were incubated in 10% FBS for 18 h and supernatants assayed for IFN activity
[c] 4×10^5 NPC were incubated for 3 h in 96-well tissue-culture plates in 20% FBS, then washed four times with warm medium to remove nonadherent (NA) NPC; medium containing 10% FBS was added and incubated 18 h. Supernatants from adherent (AD) NPC (more than 90% macrophages) were assayed for IFN activity
[d] The yield of NA-NPC recovered from nylon wool was 30%–35%; viability: 90%
[e] 20×10^6 NPC were treated with a final dilution of 1:300 α-Thy1.2 antibody for 30 min at 4°C, washed and treated with a 1:40 final dilution of baby rabbit complement (C) for 60 min at 37°C. The cells were washed, adjusted to 4×10^5 cells per well in RPMI 1640 medium containing 10% FBS, incubated for 18 h and assayed for IFN activity. Reagents (silica, carbonyl iron or α-Thy1.2 antibody) alone did not effect the IFN assay
[f] NPC were treated with silica, carbonyl iron or α-Thy1.2 antibody and then incubated with Ab-^{51}Cr-SRBC for 4 h and assayed for phagocytic activity, as described in Materials and Methods. Reagents alone had no effect on chromium release from Ab-^{51}Cr-SRBC.
One of three representative experiments. NT, not tested

(1 mg/ml), an agent that selectively destroys macrophages [22], resulted in complete abrogation of both IFN activity and phagocytic activity (Table 2, expt A). The removal of phagocytic cells from NPC by carbonyl iron treatment also inhibited both activities (Table 2, expt B). NPC were also fractionated according to their adherence properties. NPC adherent to plastic (more than 90% macrophages on the basis on uptake of latex beads) produced IFN activity comparable to that of unfractionated NPC (Table 2, expt C), whereas NPC not-adherent to nylon wool (less than 5% macrophages) did not produce detectable amounts of IFN. NPC were also treated with anti-Thy1.2 antiserum and complement specifically to eliminate T cells. As shown in Table 2 (expt D), this treatment did not affect the ability of NPC to produce IFN. These experiments demonstrate that silica-sensitive, carbonyliron-sensitive, phagocytic, adherent, and Thy 1.2$^-$ cells were the source of IFN within the NPC population.

Type of IFN produced by NPC

The type of IFN produced by NPC was determined by either directly treating the cell-free culture supernatant with an appropriate antiserum to IFN before the IFN assay or by adding the antiserum to the cells and culturing them prior to the IFN assay. As shown in Table 3, addition of anti-IFNα/β to supernatants from NPC, almost completely neutralized the IFN activity (from 544 units/ml to 39 units/ml), whereas antiserum against IFNγ had no effect on IFN activity. Incubation of antiserum against IFNα/β with NPC for 18 h gave similar results. Therefore, the IFN produced by NPC, cultured in 10% FBS, had the antigenic properties of IFNα/β.

Requirements necessary to produce IFN by NPC

The presence of IFN activity in NPC supernatants from conventional C3HeB/FeJ mice suggested that IFN was produced spontaneously. However, the addition of either actinomycin D and cycloheximide, inhibitors of RNA and protein synthesis, abrogated the ability of NPC to produce IFN (Table 4). Since endotoxin is a ubiquitous contaminant in many biological materials, including FBS, and at the same time a potent IFN inducer in murine macrophage cultures [11], the possibility that extremely low concentrations of endotoxin in the tissue-culture medium or FBS acted as a stimulus for IFN production by NPC was investigated. To determine whether endotoxin in FBS was responsible for IFN production, NPC from endotoxin-responsive C3HeB/FeJ mice were cultured in varying concentrations of FBS or albumin. Additionally, polymyxin B, an agent known to neutralize the biological activity of endotoxin by binding to the active lipid A moiety [27], was added to FBS-containing medium and NPC were subsequently cultured. As shown in Table 5, NPC cultured in 10% FBS (containing a final concentration of 24 pg/ml endotoxin as assessed by *Limulus* lysate assay) produced

Table 3. Type of IFN produced by NPC

Treatment	IFN (units/ml)			
	Reference IFNα/β	Reference IFNγ	supernatants from NPC+ antiserum[a]	NPC+ antiserum[b]
None	171	377	544	261
α-IFNα/β	<20	403	39	<20
α-IFNγ	185	<20	546	249
α-IFNα/β+α-IFNγ	<20	<20	NT	<20

[a] 4×10^5 NPC were cultured in RPMI 1640 medium containing 10% FBS for 18 h and their supernatants obtained. Supernatants were treated with either α-IFNα/β at a final concentration of 1:260 (a dilution that effectively neutralizes the antiviral activity effect of at least 500 units/ml IFNα/β) or α-IFNγ at a final concentration of 1:50 (a dilution that effectively neutralizes the antiviral activity of at least 500 units/ml IFNγ)
[b] 4×10^5 NPC were cultured in RPMI 1640 medium containing 10% FBS and either α-IFNα/β at a final concentration of 1:260 or α-IFNγ at a final concentration of 1:50. After 18 h, supernatants were obtained and assayed for IFN activity
One of two representative experiments. NT, not tested

Table 4. Protein synthesis requirement for IFN production by NPC

Treatment of NPC[a]	Concentration (μg/ml)	IFN (units/ml)
None		279
Actinomycin D	1.0	<20
	0.1	64
	0.01	191
Cycloheximide	100	<20
	10	<20
	1	<20

[a] 4×10^5 NPC were incubated in RPMI 1640 medium with 10% FBS and various concentrations of actinomycin D or cycloheximide added for 1 h, cells washed three times and fresh medium added. After 18 h, culture supernatants were tested for IFN activity. Viability of cells was not affected by either treatment. One of three representative experiments

more IFN than NPC in 1% FBS. NPC incubated in albumin (7 pg/ml endotoxin) produced small amounts of IFN whereas NPC incubated in serum-free medium (no detectable endotoxin) did not synthesize detectable IFN. When NPC were cultured in 10% FBS or albumin with polymyxin B, no endotoxin in FBS or albumin was detected nor was IFN produced. The addition of lipopolysaccharide (1 μg/ml) to NPC, incubated in either serum-free medium or albumin, resulted in augmented IFN production. These experiments suggest that IFN production by NPC was induced by low levels of endotoxin present in FBS or albumin.

After establishing that NPC, in contrast to peritoneal exudate cells or spleen cells, produced significant amounts of IFNα/β in response to low levels of endotoxin, the question next addressed was whether the in vivo exposure of NPC to gut-derived endotoxin could be the cause of NPC "priming". Therefore, IFN production by NPC from conventional C3HeB/FeJ mice was compared with age-

Table 5. IFN production by NPC under low-endotoxin culture conditions or from germ-free C3H/HeN, lipopolysaccharide-hyporesponsive C3H/HeJ mice

Expt	Strain	Culture conditions[a]	IFN (units/ml): treatment of NPC	
			none	LPS (1 μg/ml)
A	LPS-responsive C3HeB/FeJ mice	10% FBS	226	245
		1% FBS	64	NT
		0% FBS	<20	51
		10% FBS + polymyxin B 25 μg/ml[b]	<20	NT
		10% FBS + polymyxin B 5 μg/ml	<20	NT
		0.4% albumin	40	150
		0.4% albumin + polymyxin B 25 μg/ml	<20	NT
B	LPS-responsive C3HeB/FeJ mice	10% FBS	249	250
	LPS-hyporesponsive C3H/HeJ mice	10% FBS	36	40
	Germ-free C3H/HeN mice	10% FBS	60	302
C	LPS-responsive C3HeB/FeJ mice	10% FBS	160	NT
		10% FBS + polymyxin B 25 μg/ml	<20	NT
		10% PS[c]	66	NT
		10% PS + polymyxin B 25 μg/ml	<20	NT
		10% PhS[c]	<20	NT

[a] 4×10^5 NPC were cultured for 18 h under various culture conditions and supernatants assayed for IFN activity. Viability of NPC after 18 h of incubation in serum-free medium was 25–50%, as assessed by trypan blue exclusion. Viability in 1% FBS and albumin was 75%–80% and did not differ from viability in 10% FBS. Final concentration of endotoxin (as described in Materials and Methods) in medium with 10% FBS and albumin was 24 pg/ml and 7 pg/ml respectively
[b] Polymyxin B which was added to FBS or albumin prior to addition of cells had no affect on the viability of cultured NPC (as assessed by trypan blue exclusion). Endotoxin was not detected in FBS or albumin after polymyxin B treatment. Reagents, lipopolysaccharide (LPS) or polymyxin B alone did not effect the IFN assay
[c] Portal serum (PS) or peripheral serum (PhS) from C3HeB/FeJ mice.
One of three representative experiments. NT, not tested

Table 6. Role of IFN in the NK activity of NPC

Expt	Strain	Treatment		Specific release (%)		IFN (units/ml) (18 h)
		In vivo	In vitro	YAC-1 (4 h)	P815 (18 h)	
A	C3HeB/FeJ	None	None	27±1	29±2	181
			NA (nylon)[a]	50±2	2±1	<20
			AD (plastic)[a]	8±2	4±1	162
B			None	24±2	26±3	175
			Carbonyl iron[b]	22±4	0±0	<20
C			None	45±3	15±4	59
			C	42±2	13±2	53
			α-AsGm-1+C[c]	1±1	0±0	43
D			None	45±5	24±1	174
			α-IFN α/β[d]	22±1	7±1	<20
			α-IFN γ[d]	36±2	25±2	200
E		None	None	14±1	17±2	87
		poly I · poly C (−4 h)	None	38±2	20±2	57
		poly I · poly C (−24 h)	None	89±4	40±2	<20
		None	None	23±2	14±3	259
		poly I · poly C (−24 h)	C	56±2	25±2	<20
		poly I · poly C (−24 h)	α-AsGm-1+C[c]	2±1	0±0	<20
F	C57B1/6	None	None	10±1	0±0	<20

[a] 4×10^5 NPC, nonadherent (NA) NPC or adherent (AD) NPC (more than 90% macrophages) were incubated for 4–18 h in the presence or absence of 2×10^4 ^{51}Cr-labeled tumor cells and assayed for cytotoxicity or IFN activity
[b] 4×10^6 NPC were treated with 100 mg carbonyl iron for 1 h at 37°C and a strong magnet was used to remove cells taking up carbonyl iron. Then 8×10^5 remaining cells were assayed for cytotoxicity and IFN activity
[c] 20×10^6 NPC were treated with a final dilution of 1:25 of α-AsGm-1 for 30 min at 4°C, washed and treated with a 1:40 final dilution of baby rabbit complement (C) for 60 min at 37°C. The cells were washed, adjusted to 8×10^5 cells per well in RPMI 1640 medium containing 10% FBS and assayed for cytotoxicity or IFN activity
[d] 8×10^5 NPC were incubated for 4–18 h in the presence or absence of α-IFNα/β at a final concentration of 1:260 or α-IFNγ at a final concentration of 1:50 and assayed for cytotoxicity or IFN activity

and sex-matched NPC from germ-free C3H/HeN mice. As shown in Table 5, NPC from germ-free C3H/HeN mice produced less IFN than conventional mice (60 units/ml and 249 units/ml, respectively). When lipopolysaccharide was added in vitro to NPC from germ-free mice, the amount of IFN increased dramatically (60 units/ml to 302 units/ml), whereas the level of IFN synthesized by NPC from conventional mice did not change. The synthesis of IFN by NPC from endotoxin-hyporesponsive C3H/HeJ mice, cultured in 10% FBS, was also determined. As shown in Table 5, NPC from endotoxin-hyporesponsive C3H/HeJ mice produced less IFN than NPC from age- and sex-matched endotoxin-responsive C3HeB/FeJ mice (36 units/ml and 249 units/ml, respectively). Furthermore, the addition of lipopolysaccharide (1 μg/ml) to NPC from C3H/HeJ mice did not increase IFN production. In another experiment (Table 5), NPC incubated in 10% portal serum produced detectable levels of IFN (66 units/ml), which was inhibited by polymyxin B, while NPC incubated in 10% peripheral serum did not produce detectable IFN.

Table 7. IFNα/β can activate nonadherent NPC to lyse P815 tumor cells[a]

Cells	Additions[b]	Specific release (%) P815 (18 h)	IFN (units/ml)
NPC	None	11±1	278
NA-NPC	None	0±0	<20
NA-NPC	IFNα/β	10±3	250
Spleen	None	1±0	<20
NA-spleen	None	0±0	<20
NA-spleen	IFNα/β	19±7	250

[a] 4×10^5 NPC or nonadherent (NA) NPC were incubated for 18 h in the presence or absence of 2×10^4 ^{51}Cr-labeled tumor cells and assayed for cytotoxicity or IFN activity. The yield of NA-NPC recovered from nylon wool was 30%–35%; viability: 90%
[b] 250 units/ml IFNα/β was added at the initiation of 18 h assay

Role of IFNα/β in augmentation of liver-associated natural killing activity

Previously this laboratory [3] has shown that NPC from C3HeB/FeJ mice can lyse both the standard NK target, YAC-1 tumor cells, in 4 h and the NK-resistant target, P815 tumor cells, in 18 h [32]. Since IFNα/β can augment NK activity against YAC-1 cells and induce non-MHC-restricted lytic activity against P815 cells [33], experiments were conducted to determine what effect Kupffer cells (NPC adherent to plastic) production of IFNα/β had on NPC lytic activity (Table 6). NPC were fractionated on nylon wool columns prior to assay against YAC-1 (4 h) and P815 tumor cells (18 h). Cells not adherent to nylon wool have been shown to be enriched in NK cells and depleted of macrophages [12]. NPC not adherent to nylon

wool displayed augmented cytotoxic activity against YAC-1 cells but no cytotoxic activity against P815 cells compared with unfractionated NPC. Again, no IFN was produced by these NPC not adherent to nylon wool. Kupffer cells did not lyse either tumor cell but produced IFN (Table 6A). The removal of Kupffer cells from NPC by carbonyl iron treatment led to loss of cytotoxic activity against P815 cells and IFN activity while retaining cytotoxic activity against YAC-1 cells (Table 6B). Treatment of NPC with anti-asialo GM1 and complement, which eliminates NK activity [18], abrogated the cytotoxic effects of NPC against YAC-1 and P815 target cells, but did not inhibit IFN production (Table 6C). However, the same treatment did not inhibit the ability of NPC to lyse WEHI-164 cells, the target of natural cytotoxic cells [34] (data not shown). The addition of anti-IFNα/β to NPC inhibited lysis of P815 by 80% (24% to 7%). Anti-IFNγ had no effect on lysis of either tumor cell (Table 6D). The addition of unlabeled tumor cells to NPC did not influence IFN production (data not shown). These experiments confirm that NK cells within the liver were the effector cells against YAC-1 cells. Furthermore, the interaction of liver-associated non-MHC-restricted effector cells and IFNα/β was necessary to induce lytic activity against P815 tumor cells.

NPC from polyI·polyC-treated mice did not produce of IFN. However, these cells displayed augmented cytotoxic activity against both tumor cells (Table 6E) compared to NPC from untreated mice. Furthermore, this augmented cytotoxic activity was abrogated by treatment of NPC with anti-asialo-GM1 and complement. NPC from C57Bl/6J mice, which produced low amounts of IFN, were also assayed for cytotoxicity against both target cells. As shown in Table 6F, NPC from this strain inefficiently lysed YAC-1 target cells and did not kill P815 cells. Finally, to demonstrate further that IFNα/β was required for liver-associated non-MHC-restricted effector cells to lyse P815 tumor cells, NPC not adherent to nylon wool were mixed with IFNα/β (250 U/ml) and cytotoxic activity against P815 cells was determined. As shown in Table 7, these cells could lyse P815 tumor cells. IFNα/β did not directly lyse P815 cells (data not shown).

Discussion

Murine Kupffer cells (adherent, phagocytic, carbonyl-iron-sensitive, silica-sensitive NPC) from C3HeB/FeJ mice produce significant amounts of IFNα/β in vitro in response to low levels of endotoxin present in FBS-containing medium. In contrast, other cell populations (peritoneal exudate cells, spleen, mononuclear blood cells) from C3HeB/FeJ mice or Kupffer cells from other strains of mice (C3H/HeJ, germ-free C3H/HeN, C57Bl/6J) produce little or no detectable IFN under the same culture conditions. The amount of IFNα/β produced by Kupffer cells correlated with the ability of non-MHC-restricted effector cells in the liver to lyse NK-resistant P815 mastocytoma cells.

The evidence indicates that small amounts of endotoxin present in FBS was the stimulus for IFNα/β production by Kupffer cells from C3HeB/FeJ mice. First, production of IFNα/β was significantly diminished in medium containing 1% FBS or albumin (both of which had proportionally less endotoxin than medium containing 10% FBS) and was absent in serum-free medium. Second, neutralization of endotoxin with the antibiotic polymyxin B abrogated IFNα/β production by Kupffer cells. It could be argued that in contrast to peritoneal exudate cells and spleen cells, Kupffer cells were isolated by perfusion with collagenase, which could possibly cause some IFNα/β induction. However, the lack of IFNα/β activity in supernatants from peritoneal exudate cells incubated in vitro with collagenase argued against it being the inducing agent (Table 1). Similarly, Lepay et al. [23] have demonstrated that collagenase treatment of resident peritoneal macrophages had no effect on their oxidative activity.

Kupffer cells from C3HeB/FeJ mice, incubated in 10% FBS, but not in albumin, were refractory to exogenous stimulation of IFNα/β by lipopolysaccharide (Table 5). Most probably the endotoxin level in FBS already exceeded the sensitivity threshold of Kupffer cells, above which they do not respond in a dose-dependent fashion to lipopolysaccharide. Similarly, Gerberick et al. [10] were unable to demonstrate any enhancing action of lipopolysaccharide on the ability of serum-conditioned alveolar macrophages to produce superoxides. The addition of lipopolysaccharide to Kupffer cells from germ-free C3H mice did augment IFN production (Table 5B). Wannemuehler et al. [39] have shown that lipopolysaccharide can restore normal immunological reactivity in gnotobiotic mice. This suggested that the natural exposure to lipopolysaccharide in vivo may be an important component of the normal maturation of the immune system of the liver.

The question arises of why Kupffer cells differ with regard to endotoxin sensitivity as compared with peritoneal exudate cells and spleen cells. The answer may lie in the fact that the liver constitutes a major reservoir of macrophages in the organism and one of its main functions is to filter portal blood incoming from the gut. Therefore, Kupffer cells are different from other macrophages, in that they are constantly exposed to endotoxin formed in the intestines from the cell walls of gram-negative bacteria [14, 25, 28]. In support of this possibility, Kupffer cells cultured in portal serum but not in peripheral serum produced IFN (Table 5C). According to Vogel [37], endotoxin may serve in vivo as an endogenous macrophage differentiation signal, advancing macrophages to a more differentiated state, resulting in macrophage 'activation'. The fact that Kupffer cells from endotoxin-hyporesponsive C3H/HeJ or germ-free C3H/HeN mice synthesized much less IFNα/β than Kupffer cells from endotoxin-responsive C3HeB/FeJ mice, when incubated under the same culture conditions (Table 5B), also supports this view. However, other factors beside gut-derived endotoxin may play a role in the 'priming' of Kupffer cells, since some IFN production from germ-free mice was detected.

Previous studies by Virelizier and Gresser [36] have shown that C3H mice were resistant to mouse hepatitis virus-3 (MHV-3) whereas C57Bl/6 mice are susceptible to this infection. Furthermore, they have suggested that IFNα/β is an important host factor in the initial response of mice to MHV-3 infection. Injection of anti-IFNα/β into C3H mice at the onset of infection led to both increased liver damage and death. The data presented here are consistent with the notion that Kupffer cells from C3HeB/FeJ mice may constitutively produce IFNα/β in response to gut-derived endotoxin present in portal serum. The local IFN production by Kupffer cells from some strains of mice may prevent or lessen viral infection. Even though

IFN may be constitutively produced by the liver, it is not likely to be detected in the serum because peripheral serum from C3H mice contains high levels of circulating neutralizing antibodies to IFNα/β [6].

Decker et al. [5] have previously reported that NPC from C57Bl/6 mice have low cytotoxic activity against YAC-1 cells and no activity against P815 cells. The data reported here also confirm this observation. Furthermore, it seems the reason for low NK activity by NPC from C57Bl/6 mice against YAC-1 cells is due to the small amount of IFNα/β produced by their Kupffer cells (Table 1). It has been reported that IFNα/β can augment NK activity [33]. Therefore, it was reasonable to expect that Kupffer cells from C3HeB/FeJ mice, which produce high amounts of IFNα/β, could augment liver-associated NK activity against YAC-1 cells. IFNα/β activated NPC from C3HeB/FeJ could also lyse NK-resistant P815 cells in 18 h. It is uncertain if these cells are classical NK cells or are a unique subset of effector cells within the liver, which mediate cytotoxicity without MHC restriction [21]. It can not be entirely ruled out that other mediators produced by the liver can also directly or indirectly augment liver-associated lysis. When anti-IFNα/β was added to NPC, antiviral IFN activity was totally abrogated although the cytotoxic activity against P815 cells was greatly reduced (24% to 7%) but not eliminated. Other mediators, such as interleukins 1 and 2, can boost NK activity [7]. In preliminary experiments, supernatants from NPC contained detectable amounts of interleukin-1 activity but not interleukin-2 activity (data not shown). In other experiments, Keller et al. [19] have shown that Kupffer cells were much more efficient than peritoneal macrophages in their cytostatic effect against proliferating tumor cells. It is quite possible that IFNα/β was responsible for this antiproliferative effect of Kupffer cells.

Interestingly, the administration of the IFN inducer polyI·polyC to C3HeB/FeJ mice induced systemic IFNα/β and augmented liver-associated NK activity while at the same time downregulating IFNα/β production by Kupffer cells. This suggested that either poly I·polyC caused the migration of NK cells into the liver from other sites or that systemic IFNα/β can directly augment liver-associated NK activity and at the same time reduce Kupffer cell production of IFNα/β. The IFN response of cells to IFN inducers can be modified by prior cellular production of IFN [35]. A state of hyporesponsiveness or inhibition of IFN has been observed following administration of polyI·polyC [1].

IFNα/β may have other biological effects on cells in the liver besides its anti-viral and anti-tumor function. It may downregulate expression of Ia antigen and Mac-1 expression on Kupffer cells [13, 38], prevent the secretion of reactive oxygen intermediates by these cells [23], and decrease cytochrome-*P*-450-dependent drug transformation in hepatocytes [2].

Acknowledgements. We thank Paul Evans for his technical assistance and Diane Moreno for performing the *Limulus* amoebocyte lysate assay. We also thank Dr. Eugene Sulkowski and Dr. Boris Albini for their valuble discussions.

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Received April 27, 1988/Accepted July 1, 1988