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Production by Mixed Lymphocyte Cultures of a Type II Interferon Able to Protect Macrophages Against Virus Infections

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In supernatants of mixed mouse spleen cell cultures established for 4 days, a species-specific inhibitor of virus replication with a broad antiviral spectrum was found. The inhibitor was destroyed by trypsin, was nondialyzable and acid labile, and was not neutralized by antibody to mouse L cell interferon. This indicates that in mixed lymphocyte cultures a type II interferon is made that has no immunological relationship with "fibroblast" interferon. This leukocyte product was shown to protect mouse peritoneal macrophages against the cytopathic effects of influenza and mouse hepatitis viruses. It is suggested that lymphocyte interferon may collaborate with macrophages in host defense against viruses, as a mediator of cellular immunity.

Evidence has accumulated that the interferon (IF) produced by leukocytes is distinct from that produced by other cells. Based principally on antigenic differences, three different types of IF can now be distinguished in mice and in humans. The first is induced in cell cultures or in fixed cells of the body; the second is induced by viruses in leukocytes; and the third is made in cultures of lymphocytes from sensitized donors after contact with specific antigens, including viral antigens, or in sensitized animals upon renewed contact with the antigen (2, 5, 7, 12, 13). The last-mentioned type of IF differs from the other two in various physicochemical properties. It has sometimes been called immune IF, and Youngner and Salvin have proposed that it should be called type II IF (17). Mixed lymphocyte cultures of mouse origin have been shown to produce IF (4). The present communication confirms the production of an IF during mixed lymphocyte reactions (MLR) and describes some properties of this IF, which show that it resembles type II IF and that it has antiviral properties in macrophages.

MATERIALS AND METHODS

Mice. Five- to eight-week-old mice of the CBA and the C57BL/10 strains were obtained from the breeding unit of the Clinical Research Centre, Harrow, United Kingdom.

Mixed lymphocyte cultures. Suspensions of mouse spleen cells were prepared by mincing the tissue with scissors and pressing the fragments through a 60-mesh stainless-steel screen. Cells were washed twice with L15 medium. The trypan blue exclusion technique showed that 98 to 99% of the leukocytes were viable at that stage. The cells were suspended in bicarbonate-buffered RPMI 1640 medium containing penicillin and streptomycin, 1% L-glutamine, and 10% fetal calf serum, decomplemented at 56°C for 30 min, known to give low background levels in mouse MLR. Except when otherwise stated, 1-ml volumes of a cell suspension containing 1.5×10^6 CBA spleen cells and 1.5×10^6 C57BL spleen cells were distributed in plastic tubes with loosely fitted caps. The cultures were incubated at 37°C in a 5% CO_2 atmosphere for 4 days. They were centrifuged at $300 \times g$ for 15 min, and their supernatants were pooled and kept at −70°C. Morphological observation showed the presence of blasts at 96 h, and incorporation of [^{3}H]thymidine (Amersham, code TRA 120), added at 72 h and measured at 96 h, was measured as published previously (8).

Mouse macrophage cultures. The peritoneal cavities of 5- to 8-week-old C57BL/10 mice were washed with 5 ml of medium 199 containing 100 U of heparin per ml. The pool of peritoneal cells was incubated for 1 h on glass cover slips. Nonadherent cells were removed by washing the cover slips three times in L15 medium. Cultures of glass-adherent peritoneal cells were placed in glass tubes on a tube holder with a 15° inclination and incubated in RPMI 1640 medium with 10% fetal calf serum.

Viruses. Murine hepatitis virus type 3 (MHV-3, Mill Hill strain) was passaged in C57BL mice and used as a 10% liver suspension, as described previously (15). The stock MHV-3 suspension used contained $10^{4.6}$ 50% lethal doses (LD_{50}) when titrated in

vivo in C57BL mice. The influenza A/Turkey/EA virus, adapted in vivo to grow in Ehrlich ascites tumor cells (6), was a gift from J. Lindenmann. It was passaged once in embryonated chicken eggs and contained 10^7 LD_{50} when titrated in vivo by intraperitoneal infection in C57BL mice. Sindbis (Egypt, Ar-339 strain), vesicular stomatitis virus (Indiana strain), vaccinia virus, and human herpes simplex type I virus were grown in chicken embryo fibroblast cultures, as described elsewhere (3).

IF assays. Except when stated otherwise, IF titrations were carried out in secondary cultures of Swiss mouse embryo cells, using a plaque reduction assay with vesicular stomatitis virus as challenge. One unit in our mouse embryo cell system was equivalent to 1 IU. IF assays included a reference IF made in Swiss mouse embryo cell cultures induced by Newcastle disease virus (NDV).

Antiserum to mouse L-cell IF. This antiserum, a gift from B. Fauconnier, was obtained by immunizing a sheep against NDV-induced mouse L-cell IF as described previously (12). This antiserum was found to have neutralizing activity against all other virus-induced tissue culture mouse IF samples tested, as well as against NDV and partially against Sendai virus-induced mouse circulating IF. When measured against 10 U of tissue culture IF, the neutralizing titer of the serum was 800.

RESULTS

Correlation between MLR reactivity and IF production. Lymphocyte proliferation and IF production in one-way MLR were measured as described in Table 1. Neither proliferation nor IF production was observed when autologous spleen cells were mixed. In contrast, IF activity was constantly found in replicate supernatants of allogeneic spleen cell mixtures, showing that stimulation with foreign H2 antigens is necessary for IF production during MLR.

Interferon activity of MLR supernatants. Pooled supernatants of 4-day-old MLR were tested for IF activity in mouse embryo fibroblast cultures. Antiviral activity was found against the four viruses tested (Table 2). For comparison, our internal reference IF was included and showed similar variations of titers depending on the challenge virus. The species specificity of MLR-IF was demonstrated by lack of antiviral activity in either chicken or monkey (Vero) cells.

Characterization of MLR-IF. MLR-IF was nondialyzable and trypsin sensitive, features shared by all interferons (Table 3). MLR-IF, unlike type II IF (17), was relatively thermolabile. However, it shared with type II IF a property considered to be characteristic, namely, inactivation at pH 2. Antibody against L-cell IF did not neutralize at all the activity of MLR-IF, which confirms that the two IF types are distinct (Table 4).

Antiviral effects of MLR-IF in mouse macrophage cultures. The effects of MLR-IF were tested against two viruses that replicate in mouse macrophages with cytopathic effects (CPE). MHV-3 produces distinctive multinucleated giant cells that increase in size so that eventually nearly all the cells in the culture are involved, and the degree of CPE is closely paralleled by the amount of virus replication, as we have previously shown (14). The strain of influenza virus used rapidly destroys the cells,

TABLE 2. *Antiviral activity of MLR supernatant*

Cell type	Challenge virus[a]	IF titer (U/ml)[b]	
		MLR supernatant	Reference IF
Mouse embryo	VSV	150	47
	HSV	250 (1.6)[c]	65 (1.2)
	Sindbis	310 (2.0)	95 (2.0)
	Vaccinia	20 (0.13)	6 (0.13)
Chicken embryo	VSV	0	0
Vero (monkey)	VSV	0	0

[a] VSV, Vesicular stomatitis virus; HSV, herpes simplex virus.

[b] IF titrations were performed for each virus tested using a plaque reduction assay as described in Materials and Methods.

[c] Numbers in parentheses show the ratio of activity against the virus tested as compared with that against VSV.

TABLE 1. *Correlation between MLR reactivity and IF production*

Origin of spleen cells		Thymidine incorporation per culture (cpm at day 4)[a]	IF titers[b] in supernatants at day 4
Responding	Stimulating		
C57BL/6	C57BL/6 (+1,000 rads)	8,821 (3,449–12,363)	0
C57BL/6	DBA2 (+1,000 rads)	237,423 (152,342–322,505)	34 (30–45)[a]

[a] Range of results in triplicate cultures is indicated in parentheses.

[b] IF titrations were performed in monolayers of L929 murine cells as previously described (16). The sensitivity of this assay is about 10-fold less than that of the plaque reduction assay in mouse embryo fibroblasts described in Materials and Methods.

TABLE 3. *Some properties of MLR-IF*

Treatment	IF activity
None	450
Dialysis[a]	
pH 7.35	407
pH 2	0
Heating (56°C, 20 min)[b]	54
Trypsin (0.6 μg/ml, 30 min, at 37°C	0

[a] One milliliter of undiluted MLR-IF was dialyzed overnight against 20 ml of either phosphate-buffered saline at pH 7.35 or Sörensen glycine buffer at pH 2.00, with continuous stirring.

[b] Five-tenths milliliter of undiluted MLR-IF was immersed for 20 min in a water bath at 56°C.

TABLE 4. *Lack of effect of anti-mouse L-cell-IF serum on MLR-IF*

Expt	Prepn tested	Serum[a]	Residual activity (U/ml)
1	Mouse embryo fibroblast IF	Normal sheep	66
	Mouse embryo fibroblast IF	Anti-L-cell IF	0
	MLR-IF	Normal sheep	115
	MLR-IF	Anti-L-cell IF	125
2	MLR-IF	Normal sheep	90
	MLR-IF	Anti-L-cell IF	100

[a] Final concentration of anti-L-cell-IF serum was 1/10 in experiment 1 and 1/20 in experiment 2. Titration of antiviral activity was carried out immediately after a 1-h incubation of IF with the serum at 37°C.

which become round and detach from the cover slip on which they are cultured. Macrophage cultures incubated for 24 h with MLR-IF were completely protected against 10 mean infectious doses (ID_{50}) of the highly virulent MHV-3 and considerably protected against 100 ID_{50} of the virus. Similarly, macrophages were protected by MLR-IF against 1,000 ID_{50} of the virulent influenza virus.

DISCUSSION

The observations presented here show that MLR-IF is distinct from tissue culture IF in its acid sensitivity and susceptibility to neutralization by specific antibody. The properties of MLR-IF resemble those of type II IF generated in bacillus Calmette-Guerin-sensitized mice challenged with tuberculin (17). In the present study, the production of type II IF resulted from contact of spleen cells with foreign H2 antigens. In both cases, lymphocytes, responding to an antigenic stimulus, appeared to be able to produce a particular type of IF molecule. It could be argued that macrophages, which are known to be present among cultured spleen cells, might be responsible for IF production. Indeed, Rasmussen et al. demonstrated that the presence of macrophages is necessary for optimal IF production in cultures of stimulated human leukocytes (11). However, Valle et al. showed that lymphocytes, but not macrophages, produce IF in combined macrophage-lymphocyte cultures stimulated with herpes simplex antigen (13). The possibility thus exists that lymphocyte IF, as a mediator of cellular immunity, might be operative in host defense against viruses. However, the precise mechanism by which IF could be protective is still unknown.

Macrophages play a special role in host defense against viruses. If viruses are able to replicate in macrophages, they are likely to be virulent in adult animals and to produce CPE in target organs such as the liver. Indeed, we have shown a striking correlation between the ability of MHV-3 to replicate in macrophages and the susceptibility, resistance, or semiresistance of various strains of mice to MHV-3 infection (14). In sites of virus replication, mononuclear cell infiltrates are often found (1). Hence, factors that can limit virus infection in macrophages deserve careful attention. Evidence has been presented that cell-mediated immunity and mononuclear phagocytes may collaborate in the resolution of certain virus infections, for example, in herpes simplex virus infection (10). One way in which this collaboration between lymphocytes and macrophages may occur is by the liberation of IF from lymphocytes. In our hands, MLR-induced type II IF was effective in limiting virus replication and CPE, not only in fibroblasts but also in mononuclear phagocytes. Fibroblast IF is also able to induce protection against viruses in macrophages, suggesting that most substances with IF activity have this capability. However, our observation that a product of an in vitro immune reaction has an antiviral effect in macrophages

TABLE 5. *Effect of MLR-IF on virus-induced CPE in mouse macrophage cultures*

Cultures	Degree of CPE with infecting virus:					
	MHV-3		Influenza A/Turkey/EA			
	10^{1}[a]	10^{2}	10^{1}	10^{2}	10^{3}	10^{4}
Control	++[b]	+++	++	+++	+++	+++
MLR-IF treated[c]	0	+	0	0	0	+

[a] Virus infecting dose.

[b] CPE observed 48 h after infection were graded as follows: 0, none; +, <25% of cells affected; ++, 25 to 50% of cells affected; +++, <50% of cells affected.

[c] Macrophage cultures were incubated for 24 h with 1 ml of undiluted supernatant of MLR (450 IF units/ml) or with a control medium containing an identical percentage of the same batch of fetal calf serum.

provides a mechanism by which lymphocytes and macrophages could cooperate in terminating infections during the course of an immunological response in vivo. This mechanism could be especially important in infections such as established MHV-3 or herpesvirus infections, where antibody alone does not readily confer passive protection (9, 10).

Lymphocyte IF also affects immune responses. We have shown that mouse serum containing type II IF can depress primary in vitro immune responses (A. C. Allison, E. Chan, and J. L. Virelizier, in preparation) and also that the levels of circulating IF parallel the effects of virus infection to an unrelated antigen (16). Thus, IF production by lymphocytes could modify immune responses to virus antigens themselves, a factor that has to be considered when analyzing the effectiveness of the response. In this respect, as well as in host defense against viruses, type II IF might be a major mediator of cellular immunity.

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