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PRIMING AND TRIGGERING OF TUMORICIDAL AND SCHISTOSOMULICIDAL MACROPHAGES BY TWO SEQUENTIAL LYMPHOKINE SIGNALS: INTERFERON-γ AND MACROPHAGE CYTOTOXICITY INDUCING FACTOR 2[1]

PETER H. KRAMMER,[2]* CLAIRE F. KUBELKA,† WERNER FALK,* AND ANDREAS RUPPEL†

*From the *Institute for Immunology and Genetics of the German Cancer Research Center, and the †Institute for Immunology and Serology of the University, Heidelberg, Federal Republic of Germany*

We describe a new lymphokine activity, macrophage cytotoxicity inducing factor 2 (MCIF2), in the T cell mitogen-induced supernatant of a murine T cell clone in long-term culture. MCIF2 has the following properties: 1) it elutes from a Sephadex G-100 column in three m.w. forms (10, 34, and 100 KD); 2) it is acid labile (pH 2 to 4) and heat sensitive (80 min at 56°C); 3) it is not constitutively secreted, coexists in the same supernatant with immune interferon (IFN-γ), and synergizes with IFN-γ for induction of tumoricidal and schistosomulicidal resident peritoneal mouse macrophages. We uncoupled this synergy and show that IFN-γ serves as the first ("priming") and MCIF2 as the second ("triggering") signal for macrophage activation. Application of the lymphokines in the reverse order was ineffective. These data demonstrate a two-step mechanism of macrophage activation.

Macrophages activated by T cell-derived lymphokines, called macrophage-activating factors (MAF),[3] play an essential role in the defense against cancer and infectious diseases (1, 2). MAF are a heterogeneous group of lymphokines and induce a variety of macrophage functions (3–5), e.g., the killing of tumor cells and of schistosomula of *Schistosoma mansoni* in vitro (3, 4, 6–8).

Recently, immune interferon (IFN-γ) was characterized as an important MAF in the supernatant (SN) of normal and malignant T cells from mouse and man (9–23). Macrophages stimulated with IFN-γ increase the expression of Ia antigens on the cell membrane, show a higher oxidative metabolism, and inhibit the replication of intracellular microorganisms (17, 20, 22, 23). IFN-γ induces tumoricidal activity, particularly of "elicited" macrophages and in the presence of costimulators like lipopolysaccharide (LPS) (9, 10, 15, 16). Activation of tumoricidal resident peritoneal mouse macrophages by IFN-γ alone, however, is suboptimal and variable (9, 10, 15, 16), or totally ineffective in LPS-low-responder mice (24).

These data and other data in the literature on non-IFN-γ MAF (25, 26) prompted us to investigate whether other T cell-derived lymphokines in place of LPS and combined with IFN-γ can activate macrophages. Our experiments showed synergy of IFN-γ and another T cell-derived lymphokine for induction of macrophage tumoricidal activity. This lymphokine is secreted by normal T cells, long-term T cell clones, and T cell hybridomas and was called macrophage cytotoxicity inducing factor 2 (MCIF2). In most cases MCIF2 coexisted with IFN-γ in the SN of T cells (16).

In the experiments reported in this paper we show that MCIF2 in the SN of the long-term T cell clone 29 elutes from a Sephadex G-100 column in three m.w. forms (10 kilodaltons [KD], 34 KD, and 100 KD), is acid labile, heat labile, and relatively alkaline stable. In addition, we show the synergy of MCIF2 in the IFN-γ-depleted SN of T cell clone 29 with IFN-γ for activation of tumoricidal and schistosomulicidal mouse macrophages. Finally, we demonstrate that activation of resident mouse macrophages occurs via a two-step mechanism with IFN-γ as the first and MCIF2 as the second signal.

MATERIALS AND METHODS

Mice. DBA/2 (H-2^d) mice were obtained from the Zentralinstitut für Versuchstiere, Hannover, F.R.G. Mice of both sexes were used for experiments at 6 to 8 wk of age.

Culture medium. Cells were cultured in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with L-glutamine (2 mM final concentration), streptomycin (100 μg/ml), and penicillin (100 U/ml), HEPES buffer (25 mM final concentration), 2-mercaptoethanol (10^{-5} M), and 10% fetal calf serum (FCS).

T cell clone 29. The establishment, culture, and induction of lymphokine secretion of T cell clone 29 has been described (4, 16). Briefly, C57BL/6 mice were sensitized on the abdominal skin by 2,4,6-trinitrophenyl chloride. Five days later the draining axillary and inguinal lymph node cells were cultured for 4 days. T cell clone 29 was obtained by cloning these cells in limiting dilution microcultures. The cells from this clone grow adherent to plastic, and their growth is dependent on the presence in the medium of interleukin 2 (IL 2) from SN of rat spleen cells stimulated by concanavalin A (Con A) for 24 to 48 hr. Lymphokine secretion was induced (after washing the cells in IL 2-free medium containing 2% FCS) by pulsing the cells in a large tissue culture flask (Falcon bottles, flat 3024F; Falcon, Oxnard, CA) for 4 hr with 10 μg/ml Con A, washing three times with 20 ml medium, and culturing for 24 hr in medium free of both IL 2 and Con A. After the incubation, the SN was harvested, centrifuged, and stored at −20°C. Sham-induced SN was prepared without Con A stimulation.

Several additional IFN-γ-positive control SN from normal T cell clones, induced with Con A as described above, were shown to be negative for the lymphokine synergizing with IFN-γ (16). Furthermore, the maximal amount of Con A remaining in the SN after Con A pulse induction, using trace-labeled Con A, was less than 0.01

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[2] Correspondence should be addressed to Dr. Krammer at: Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 6900 Heidelberg, Federal Republic of Germany.
[3] Abbreviations used in this paper: MAF, macrophage-activating factor; SN, supernatants; KD, kilodalton.

μg/ml (27). Con A at a concentration of 1 μg/ml, however, did not induce macrophage activation (data not shown). Therefore, an effect of Con A in our experiments on induction of tumoricidal or schistosomulicidal macrophages could be excluded.

Depletion of T cell clone 29 SN of IFN-γ. T cell clone 29 SN was depleted from IFN-γ by solid-phase absorption on protein A-Sepharose coupled with the monoclonal hamster anti-mouse IFN-γ antibody H22.1 (28). An equivalent of 1 ml slurry of Sepharose 4B was coupled by cyanogen bromide with 1 mg protein A (Pharmacia, Uppsala, Sweden) (29). Protein A-Sepharose (400 μl), diluted 1/15 in phosphate-buffered saline (PBS), was suspended in small plastic microtubes (Eppendorf No. 3810; Netheler and Hinz, Hamburg, F.R.G.), coated with 1% bovine serum albumin (BSA), washed with PBS, and incubated under gentle shaking for 1 hr at room temperature with 1600 ng/tube H22.1 or control antibodies, respectively. After the incubation, the antibody-coupled protein A-Sepharose was washed twice with culture medium and 2% FCS and used for absorption of prediluted T cell clone 29 SN at room temperature under gentle shaking for 1 hr. SN were harvested after centrifugation of the tubes at 1600 × G for 10 min, UV light-sterilized, and used for macrophage activation.

Column chromatography of T cell clone 29 SN. One milliliter of T cell clone 29 SN was chromatographed on a 38 x 1.5 cm Sephadex G-100 (Pharmacia, Uppsala, Sweden) column in PBS at a flow rate of 1.6 ml/hr. Human immunoglobulin G, BSA, and cytochrome *c* were used as m.w. standards. One-milliliter fractions were collected, aliquoted, and tested for IFN-γ and MAF activity.

pH and temperature sensitivity of MCIF2. Aliquots of 0.5 ml IFN-γ-depleted T cell clone 29 SN (4% v/v in culture medium) were dialyzed against 50 ml of different buffers at pH 2, 4, 6, 8, and 10 overnight. The same samples were then dialyzed against 50 ml RPMI 1640 for 4 hr. In addition, 0.5-ml aliquots of IFN-γ-depleted T cell clone 29 SN were heated for 10 to 80 min at 56°C. Samples from both treatments were used as the second signal, together with IFN-γ (16 U/ml) as the first signal, for induction of macrophage tumor cytotoxicity. Untreated IFN-γ-depleted T cell clone 29 SN was used as a control.

Preparation of peritoneal macrophages. Resident macrophages were obtained from the peritoneal cavity of mice. Macrophages were enriched (>95%), as determined by uptake of carbon particles and by staining for nonspecific esterase (3), by adherence to culture plates. After 2 hr of adherence, nonadherent cells were removed and the viability of the remaining cells was determined (>95%). No lymphokines could be measured in SN from culture wells with plastic-adherent cells alone, even when these cells were stimulated with Con A (16).

Macrophage tumor cytolysis. Macrophage tumor cytolysis was tested with resident peritoneal macrophages (3×10^5 cells/culture well) without addition of costimulators, e.g., LPS, except where indicated (LPS W, *E. coli* 0127:B8; Difco Laboratories, Detroit, MI). Macrophages were incubated in 100 μl culture medium in flat-bottom microtiter plates (Costar No. 3596; Costar, Cambridge, MA) for 24 hr with or without MAF-containing T cell clone 29 SN and/or IFN-γ. After removal of the SN and two washes of the cells, the culture wells were refilled with 200 μl medium, and 4×10^4 6-[^{3}H]thymidine (^{3}H-TdR)-labeled P815 (DBA/2 mastocytoma) target cells in 20 μl culture medium. The target cells (1.5×10^7 in 3 ml culture medium) were labeled with 0.2 ml ^{3}H-TdR (5 Ci/mol.; Amersham Buchler, Braunschweig, F.R.G.) for 4 hr at 37°C and washed three times in medium. After 16 hr of incubation, ^{3}H-TdR release was determined in 100 μl SN after centrifugation of the cells at 300 × G for 5 min. Radioactivity was determined in a beta counter.

Percent specific ^{3}H-TdR release was calculated by the formula

$$\%\ \text{Specific}\ ^3\text{H-TdR release} = \left[\frac{\text{experimental cpm} - \text{control cpm}}{\text{maximum cpm} - \text{control cpm}}\right] \times 100$$

Experimental cpm are cpm released in wells containing activated macrophages and P815 tumor target cells; control cpm are cpm released from P815 tumor target cells cultured with nonactivated macrophages; maximum cpm were cpm from labeled P815 tumor target cells (4×10^4) released by lysis with Triton X-100 (5% v/v in H_2O). The control cpm were less than 15% of the maximum cpm, and replicate cpm showed less than 5% difference from the mean.

Macrophage schistosomula killing. Schistosomula of *Schistosoma mansoni* from a locally maintained Puerto Rican strain (30) were obtained in vitro by penetration of isolated mouse skin by using conditions described previously (31). The assay for schistosomula killing was performed by culturing macrophages (3×10^5/culture well) with and without MAF-containing T cell clone 29 SN and/or IFN-γ. After 24 hr, the incubation medium was removed and replaced by a schistosomula suspension containing an average of 50 larvae (effector to target ratio of 6000:1). The viability of schistosomula was visually determined after 48 hr of culture. Controls were done with schistosomula incubated with nonactivated macrophages (3, 32).

Macrophage activation by sequential lymphokine signals. Macrophages (3×10^5/culture well) were incubated with medium (control) or lymphokines for 15 hr, gently washed twice, and further incubated with medium or lymphokines for 7 hr. After the last incubation, the cells were gently washed once and tested for tumoricidal and schistosomulicidal activity.

IFN assay and anti-IFN-γ antibodies. IFN was assayed by measuring the reduction of the cytopathic effect of vesicular stomatitis virus on L cells (4). The IFN titer is expressed as the reciprocal of the highest serial twofold dilution of the sample showing 50% reduction of the cytopathic effect. A standard reference mouse IFN-α/β (NIH standard G-002-904-511) was included in each assay. One unit (U) of IFN in the assay corresponds to 1 U of the reference IFN. Recombinant IFN-γ (*E. coli* derived, 3×10^7 U/mg protein, lot No. 2715/88, S 200, No. 3) was generously provided by Genentech, Inc., San Francisco, CA). Protein A-binding, IFN-γ-neutralizing monoclonal hamster anti-mouse IFN-γ-antibodies have been described (28). H22.1 (purified immunoglobulin) was generously provided by Dr. R. Schreiber, Scripps Clinic, La Jolla, CA.

RESULTS

Macrophage activation by recombinant IFN-γ and MCIF2 in the SN of T cell clone 29. Previous data suggested that IFN-γ alone was insufficient to induce activation of resident peritoneal mouse macrophages to kill tumor cells and schistosomula of *S. mansoni* (4, 16, 24). Therefore, we postulated a second lymphokine (MCIF2) coexisting with IFN-γ in T cell SN that synergized with IFN-γ (16).

Thus, we compared the ability of recombinant IFN-γ and of mitogen- and sham-induced SN from long-term T cell clone 29 to activate tumoricidal and schistosomulicidal resident peritoneal mouse macrophages. The mitogen-induced T cell clone 29 SN in these assays was used at a concentration at which its antiviral titer corresponded to the antiviral titer of the recombinant IFN-γ in the same test. Figure 1 shows that at the concentration used, recombinant IFN-γ and sham-induced T cell clone 29 SN were inactive, whereas mitogen-induced T cell clone 29 SN induced substantial macrophage activation in both tests. This result is in line with the literature (4, 9, 10, 16) and indicates again that IFN-γ alone is ineffective to activate resident macrophages. It should be noted that at higher concentrations (>60 to 100 U/ml IFN-γ for macrophage tumor cytotoxicity and >130 to 1000 U/ml IFN-γ for macrophage schistosomula killing) recombinant IFN-γ showed inconsistent effects, but was always negative at lower concentrations.

In each case, however, macrophage activation could be achieved with low concentrations of IFN-γ plus LPS. Under these conditions the levels of activity were similar to those obtained with T cell clone 29-derived SN (Fig. 1).

Taken together, the above data support our previous findings and indicate that IFN-γ in T cell clone 29 SN synergizes with MCIF2 for induction of tumoricidal and schistosomulicidal activity of resident peritoneal mouse macrophages. MCIF2 is not constitutively produced by T cell clone 29, but induced to be secreted by the T cell mitogen Con A.

Macrophage activation by the synergy of recombinant IFN-γ and MCIF2 in the IFN-γ-depleted T cell clone 29 SN. The fact that MCIF2 coexists with IFN-γ in the SN of T cell clone 29 makes its detection difficult. To more directly demonstrate its presence in further experiments,

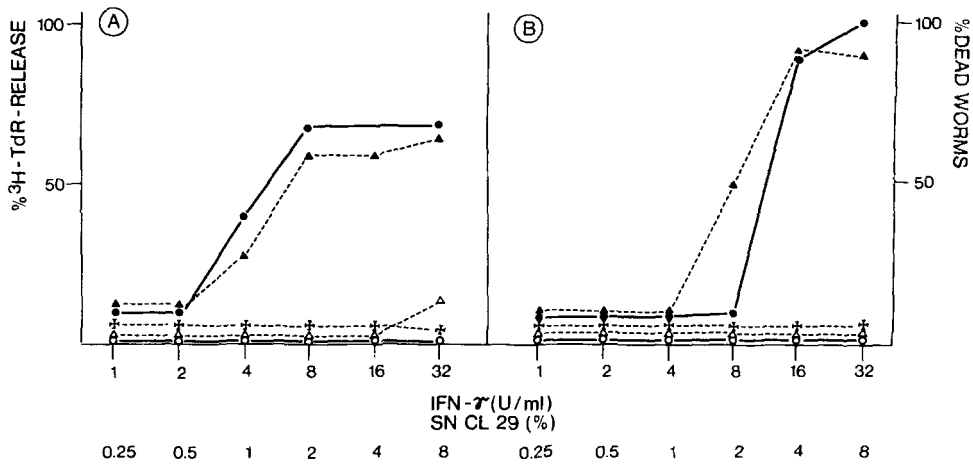

Figure 1. Activation of tumoricidal (*A*) and schistosomulicidal (*B*) resident peritoneal mouse macrophages. Macrophages (3×10^5/culture) were incubated with sham-induced T cell clone 29 SN (O—O), mitogen-induced T cell clone 29 SN (●—●), recombinant IFN-γ (Δ– –Δ), recombinant IFN-γ plus LPS (▲– –▲), and recombinant IFN-γ plus sham-induced T cell clone 29 SN (5% v/v in culture medium plus 2% FCS) (+– –+). The concentration of the mitogen-induced T cell clone 29 SN was adjusted to a concentration at which its antiviral titer corresponded to the antiviral titer of recombinant IFN-γ in the same test. After 24 hr of incubation macrophage tumor cell and schistosomula killing was determined as described in *Materials and Methods*. Similar data were obtained in three separate experiments.

we used T cell clone 29 SN depleted of IFN-γ by solid-phase absorption on protein A-Sepharose coupled with the monoclonal anti-mouse IFN-γ antibody H22.1 (H22.1 antibodies were generously provided by Dr. R. Schreiber, Scripps Clinic, La Jolla, CA). Table I shows that IFN-γ-depleted T cell clone 29 SN devoid of any measurable antiviral activity did not induce macrophage activity. MAF activity, however, was fully restored by adding it to the IFN-γ-depleted SN recombinant IFN-γ at a concentration that was completely inactive by itself. As a result, the MAF activity of the IFN-γ-depleted and recombinant IFN-γ-reconstituted T cell clone 29 SN is comparable to that of the nondepleted SN. These experiments demonstrate that IFN-γ and MCIF2 alone are both inactive for macrophage activation, but induce substantial activity when combined.

Macrophage activation by two sequential lymphokine signals: IFN-γ and MCIF2. The results described in the previous section prompted us to investigate whether the effects of IFN-γ and MCIF2 could be uncoupled, and whether macrophage activation could be induced by sequential lymphokine signals. Thus, resident peritoneal mouse macrophages were incubated with lymphokines for 15 hr, washed, and again incubated with lymphokines for 7 hr. After the second incubation, the macrophages were washed and tested for tumoricidal and schistosomulicidal activity. The lymphokines we used in these experiments were recombinant IFN-γ, mitogen- and sham-induced T cell clone 29 SN, and IFN-γ-depleted T cell clone 29 SN (devoid of antiviral and MAF activity; see Table I). Mitogen-induced T cell clone 29 SN as the first and second signal was used as a positive control (data not shown). The combination of recombinant IFN-γ with recombinant IFN-γ, or recombinant IFN-γ with sham-induced T cell clone 29 SN, respectively, used as either the first or the second signal was inactive (data not shown). Figure 2 shows that potent macrophage activation was only observed when recombinant IFN-γ was applied as the first signal and MCIF2 in the IFN-γ-depleted T cell clone 29 SN was applied as the second signal. Incubation of macrophages with lymphokines in the reverse order was ineffective. These data indicate that activation of tumoricidal and schistosomulicidal resident peritoneal mouse macrophages occurs via a two-step mechanism, with IFN-γ as the first signal and MCIF2 as the second signal.

TABLE I

Activation of tumoricidal and schistosomulicidal resident peritoneal mouse macrophages by the synergy of recombinant IFN-γ and IFN-γ-depleted T cell clone 29 SN

Addition to Culture	Macrophage Activity	
	Tumor cell killing (% ^{3}H-TdR release)	Schistosomula killing (% dead worms)
Recombinant IFN-γ[a]	8	3
T cell clone 29 SN[b]	67.4	100
T cell clone 29 SN, IFN-γ depleted[c]	0	3
T cell clone 29 SN, IFN-γ depleted and reconstituted with recombinant IFN-γ[d]	68.3	98

[a] Recombinant INF-γ was used at 24 U/ml.

[b] Control (normal Ig)-absorbed T cell clone 29 SN had an IFN-γ titer of 24 U/ml.

[c] IFN-γ in T cell clone 29 SN (used at the same concentration—4% v/v in medium—as in footnote *b*) was IFN-γ depleted by absorption on the monoclonal anti-IFN-γ antibody H22.1. After absorption, the SN had no antiviral activity.

[d] IFN-γ-depleted T cell clone 29 supernatant (as in footnote *c*) was reconstituted with 24 U/ml recombinant IFN-γ. Similar data were also obtained at IFN-γ titers of 6 or 12 U/ml and were confirmed in three separate experiments.

Physicochemical characterization of MCIF2. For initial biochemical characterization of MCIF2, T cell clone 29 SN was chromatographed on Sephadex G-100. Each fraction was tested for antiviral activity in the IFN assay and at a concentration of 10% (v/v in culture medium) for induction of macrophage tumor cytotoxicity. Figure 3 shows that IFN-γ and MAF activity coeluted with a peak of activity at 34 KD (fractions 10 to 15). Again, this activity in the MAF test must have been due to IFN-γ plus MCIF2, because the highest concentration of IFN-γ in fraction 12 was only 4 U/ml, and recombinant IFN-γ alone at 10 U/ml was inactive.

To test whether IFN-γ in amounts undetectable in the IFN assay was present in the column fractions before and after the IFN-γ peak, each fraction was retested with LPS (0.5 μg/ml). No further MAF activity indicative for IFN-γ could be detected by admixture of LPS. Retest of each fraction, however, with recombinant IFN-γ (10 U/ml) revealed two further peaks of MCIF2 at 10 KD (fractions 15 to 20) and 100 KD (fractions 6 to 9).

These results are supported by the data shown in Figure 4. The fractions under each peak (peak I, fractions 7 and 8; peak II, fractions 11 to 14; and peak III, fractions 16 to 18) were pooled, and the pools were tested either with or without recombinant IFN-γ (10 U/ml) for the induction of macrophage tumor cytotoxicity. As expected,

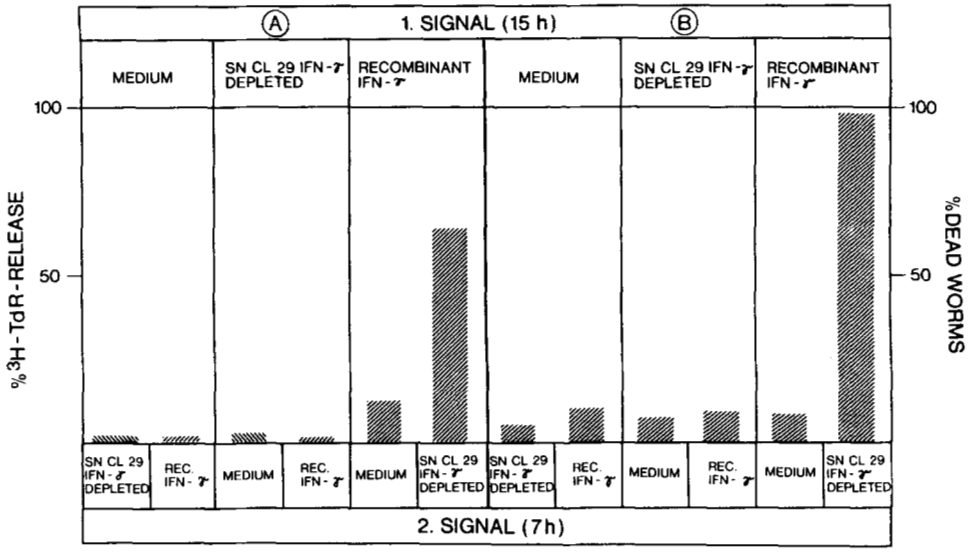

Figure 2. Activation of tumoricidal (*A*) and schistosomulicidal (*B*) resident peritoneal mouse macrophages by two sequential lymphokine signals. Macrophages (3×10^5/culture) were incubated with culture medium, IFN-γ-depleted T cell clone 29 SN (see *Materials and Methods*) (4% v/v in medium), or recombinant IFN-γ (24 U/ml) for 15 hr (first signal) or 7 hr (second signal), respectively. The macrophages were washed twice after the first and once after the second incubation. After the last wash, macrophage tumor cell and schistosomula killing was determined as described in *Materials and Methods*. Similar data were obtained with 6 and 12 U/ml IFN-γ and confirmed in three separate experiments.

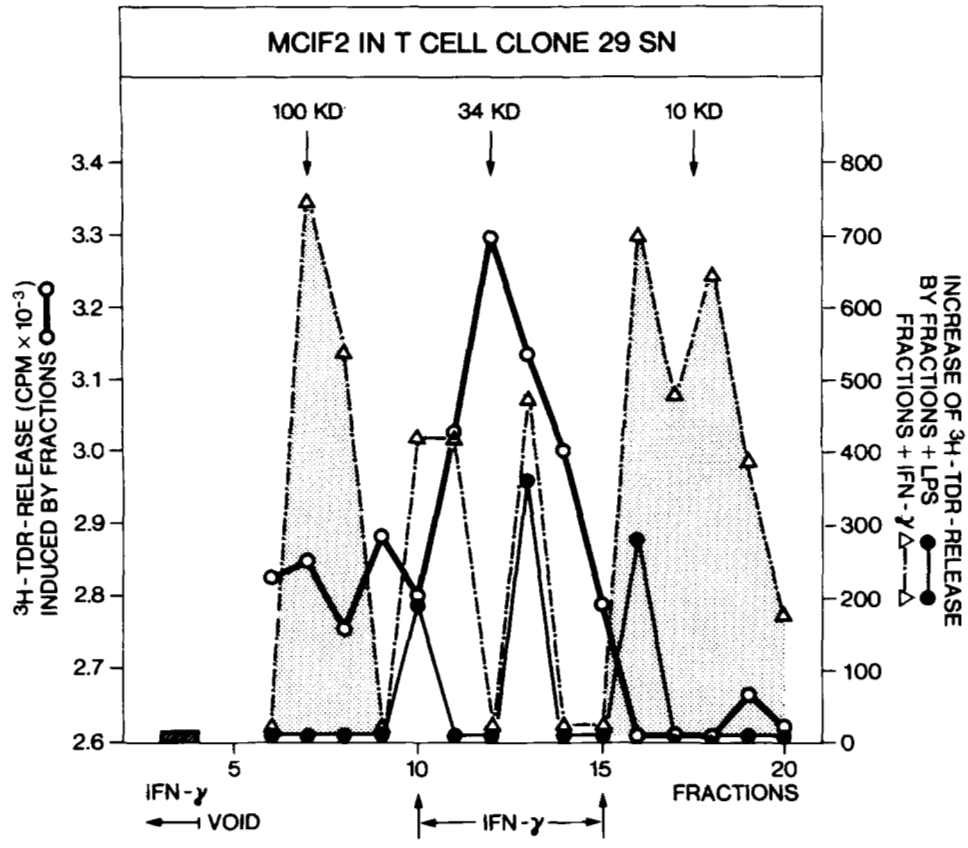

Figure 3. MCIF2 in T cell clone 29 SN. T cell clone 29 SN was chromatographed on a Sephadex G-100 column as described in *Materials and Methods*. Fractions were tested for antiviral (IFN-γ) or MAF activity (induction of tumor cytotoxicity of resident peritoneal mouse macrophages). In the MAF test, the fractions were tested at a concentration of 10% (v/v in culture medium) without any addition (○—○) or with the addition of LPS (0.5 μg/ml) (●—●) or recombinant IFN-γ (10 U/ml) (△—·—△). The cpm for recombinant IFN-γ (▨) or LPS alone were within the background cpm of macrophages incubated with culture medium alone. The shaded areas before and after the IFN-γ peak (fraction 10 to 15) represent the increase in cpm in the MAF test induced by fractions in the presence of recombinant IFN-γ over cpm induced by fractions alone. The void volume of the column is also indicated. The IFN-γ concentration of fraction 12, tested as 10% (v/v in culture medium) in the MAF test, was 4 U/ml.

direct activity without IFN-γ could only be seen with the pool under peak II (34 KD), which in addition to MCIF2 primarily contained IFN-γ. Addition of recombinant IFN-γ to this pool, therefore, did not substantially increase MAF activity. This situation, however, was different for the pool under peak III (10 KD) and under peak I (100 KD). No MAF activity was seen without, but substantial activity was seen with, recombinant IFN-γ, inactive by itself. Thus, we conclude that MCIF2 activity elutes in three peaks at 10, 34, and 100 KD.

To determine the pH and temperature sensitivity of MCIF2, IFN-γ-depleted T cell clone 29 SN was dialyzed against buffers from pH 2 to 10 and heated for 10 to 80 min at 56°C, respectively. Samples treated in this way were used as the second signal, together with recombinant IFN-γ as the first signal, to induce macrophage tumor cytotoxicity. Untreated IFN-γ-depleted T cell clone 29 SN served as a control. The results summarized in Table II show that MCIF2 is acid labile (pH 2 to 4), stable at pH 8 and 10, and inactivated by heating for 80 min at 56°C.

DISCUSSION

This paper describes the biologic and initial biochemical characterization of MCIF2, a new lymphokine activity, which plays a significant role in triggering of tumoricidal and schistosomulicidal activity of IFN-γ-primed resident peritoneal mouse macrophages. We previously

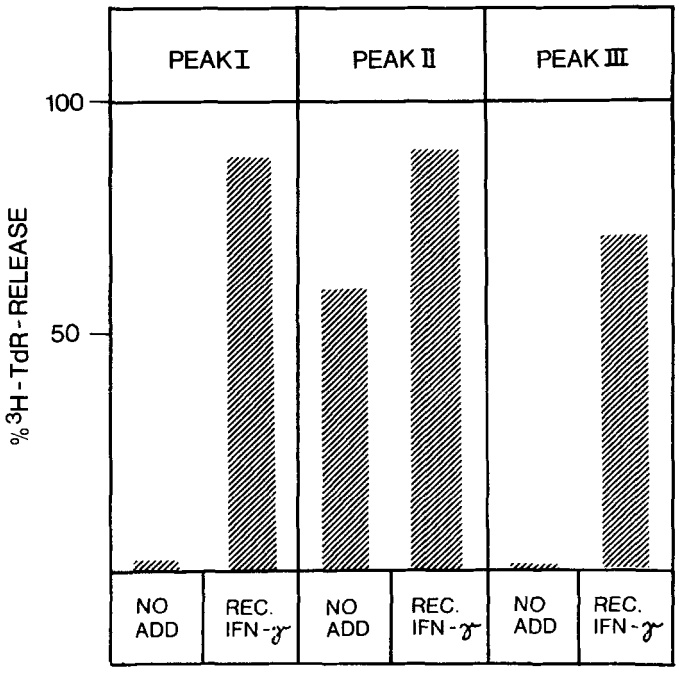

Figure 4. Induction of tumor cytotoxicity of resident peritoneal mouse macrophages by pools of fractions eluted from a Sephadex G-100 column (see Fig. 3) without addition (NO ADD) or with addition of recombinant IFN-γ (REC. IFN-γ) (10 U/ml). The peaks represent a pool of fractions 7 and 8 (peak I), fractions 11 to 14 (peak II), and fractions 16 to 18 (peak III) (see Fig. 3). Peak II contains <4 U/ml IFN-γ. The activity of recombinant IFN-γ alone was 0.6% ^{3}H-TdR release. Similar data were obtained in two separate experiments.

TABLE II

Physicochemical characteristics of MCIF2

Elution from Sephadex G-100 column[a]	10, 34, 100 KD
pH sensitivity[b]	Inactivated by pH 2 to 4
Temperature sensitivity[c]	Inactivated by 80 min at 56°C

[a] The m.w. was determined by Sephadex G-100 column chromatography of T cell clone 29 SN as described in the legend of Figure 3.

[b] IFN-γ-depleted T cell clone 29 SN (4% v/v in culture medium) as a source of MCIF2 was used as the second signal and recombinant IFN-γ (16U/ml) as the first signal to induce macrophage tumor cytolysis. Aliquots of IFN-γ-depleted T cell clone 29 were dialyzed against buffers at pH 2 to 10 overnight and 4 hr against RPMI 1640 as described in *Materials and Methods*. Inhibition of ^{3}H-TdR release with the dialyzed samples against the nondialyzed MCIF2 control (35% ^{3}H-TdR release): pH 2, 60%; pH 4, 73%; pH 6, 18%; pH 8, 0%; pH 10, 0%.

[c] MCIF2 was tested as described in footnote *b*. Aliquots of IFN-γ-depleted T cell clone 29 SN were heated for different times at 56°C. Inhibition of ^{3}H-TdR release against the nonheated MCIF2 control: 10 min 56°C, 38%; 80 min 56°C, 70%.

showed that IFN-γ and MAF activities in the SN of short-term T cell clones in limiting dilution microcultures, long-term T cell clones, and T cell hybridomas were not coordinately expressed. SN were found with low or high antiviral and the reverse MAF activity. Similar data were obtained in hybridoma SN for the induction of macrophage schistosomulicidal activity (4). The MAF activity in such SN, however, was completely inhibited by an antiserum raised against recombinant IFN-γ. Blocking of the MAF activity could be reversed by addition to the SN of recombinant IFN-γ, inactive by itself (16). Taken together, these data suggested to us that IFN-γ alone was insufficient to induce tumoricidal activity of resident peritoneal mouse macrophages. Therefore, we postulated a second lymphokine (MCIF2) coexisting with IFN-γ in the T cell SN that synergized with IFN-γ. As this synergy was also observed on the homogeneous cell population of the macrophage-like tumor PU 5-1.8 (16), it was evident that MCIF2 together with IFN-γ stimulated macrophages directly.

Previous experiments had already shown that MCIF2 activity was unlikely to be due to a contamination of the T cell SN with LPS (16). This is further made unlikely by the fact that the combination of recombinant IFN-γ with sham-induced T cell clone 29 SN, at a concentration (5%) at which the mitogen-induced T cell clone 29 SN induced plateau macrophage activity, was inactive (Fig. 1).

Meltzer (33) showed that activation of resident mouse macrophages proceeds in two steps, priming and triggering, by stimulation with two sequential lymphokine signals (33). Subsequently, Schultz and Kleinschmidt (9), and Pace et al. (10) demonstrated that the priming step was due to the activity of IFN-γ. The activity of IFN-γ-primed macrophages could then be triggered by a second signal provided by LPS. We show here that MCIF2 serves as a triggering signal for IFN-γ-primed macrophages with an efficiency that is comparable to that of LPS. MCIF2 may thus be the physiological second signal for activation of resident mouse macrophages.

The initial physiochemical characterization of MCIF2 had to take into account that activation of resident macrophages could only be achieved by IFN-γ plus MCIF2. Therefore, we had to ascertain that we characterized the properties of MCIF2 without interference from IFN-γ. Sephadex G-100 column chromatography of T cell clone 29 SN revealed a 34-KD IFN-γ peak with MAF activity. The MAF activity of this peak, however, was not due to IFN-γ alone, because recombinant IFN-γ used at higher concentrations than that of IFN-γ found under that peak was inactive by itself. We concluded, therefore, that MCIF2 was also found under the IFN-γ peak. Two additional peaks (10 KD and 100 KD) of MCIF2 activity were found in IFN-γ-negative column fractions to which we admixed recombinant IFN-γ. Thus, MCIF2 eluted from the column in three m.w. forms: 10, 34, and 100 KD. The higher m.w. forms may be aggregates of the lower ones, an observation that is not uncommon for other lymphokines, e.g., lymphotoxin (34). Although we favor the idea that MCIF2 is a lymphokine distinct from IFN-γ, we cannot exclude that IFN-γ has two different sites, an antiviral and a MAF site (28). If this were the case, the 10-KD form might be a split product of IFN-γ with an inactive antiviral site not accessible to the anti-IFN-γ monoclonal antibody H 22.1. The 100-KD form might be an aggregate of this 10-KD form.

To evaluate the pH and temperature sensitivity of MCIF2, IFN-γ-depleted T cell clone 29 SN was dialyzed against buffers at pH 2 to 10 or heated for various times at 56°C. Samples with MCIF2 treated in this way were compared with untreated MCIF2-containing, IFN-γ-depleted T cell clone 29 SN for their triggering activity on IFN-γ-primed resident peritoneal mouse macrophages. MCIF2 proved to be acid labile, heat labile, and relatively stable at alkaline pH.

Taken together, our data determine MCIF2 as a new lymphokine activity essential for triggering of IFN-γ-primed macrophages. MCIF2 may be a new molecular species, but our present data do not exclude the pleiotropic activity of a T cell-derived lymphokine that is already molecularly defined. In any case, the efficiency of macrophage activation by the combination of IFN-γ

with MCIF2 in vitro should also initiate experiments to test the efficiency of such combinations in vivo.

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