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LIPOPOLYSACCHARIDE AND POLYRIBONUCLEOTIDE ACTIVATION OF MACROPHAGES: IMPLICATIONS FOR A NATURAL TRIGGERING SIGNAL IN TUMOR CELL KILLING

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SUMMARY: There is evidence that activation of macrophages for tumor cell killing can involve either two signals (interferon/lipopolysaccharide, for example) or one signal (lipopolysaccharide or double-stranded RNA, for example). We investigated the apparent one-signal activation of bone marrow-derived macrophages for P815 mastocytoma killing by treatment with lipopolysaccharide (LPS) or by the synthetic double-stranded polyribonucleotide polyinosinic acid-polycytidylic acid (poly I:C). We found that "direct" activation of macrophages by either LPS or poly I:C was still a two-signal process. Based on antibody neutralizations, the first signal was probably mediated by LPS or poly I:C induced α/β interferon in the macrophage cultures, and the second signal was that of a direct effect of the LPS or poly I:C on the cell. The fact that poly I:C can provide the triggering signal for macrophage activation suggests a possible role for double-stranded RNA structures in macrophage triggering. Such double-stranded RNA requirements could be met by single-stranded RNAs that possess significant double-strandedness in their structures.

Several investigators have demonstrated that activation of macrophages for tumor cell killing is a two-signal process (1-3). The first signal, called priming, is usually induced by interferon (IFN), which renders the macrophages sensitive to a second-signal triggering agent such as lipopolysaccharide (LPS). Under some culture conditions macrophages can be directly activated by LPS in the absence of added IFN (4-7). Furthermore, the direct activation effects of LPS can be mimicked by the double-stranded polyribonucleotide polyinosinic acid-polycytidylic acid (poly I:C) (4,7,8). We present data in this paper which show that the direct activation of macrophages by either LPS or poly I:C is still a two-signal process. The first

ABBREVIATIONS:

LPS, lipopolysaccharide; poly I:C, polyinosinic acid-polycytidylic acid; IFN, interferon; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

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signal is mediated by LPS or poly I:C induced IFN α/β in the macrophage cultures, and the second signal is that of a direct effect of LPS or poly I:C on the cell. The demonstration that poly I:C can provide the triggering signal for macrophage activation suggests a possible role for double-stranded RNA structures as endogenous triggers for activation of macrophages for tumor cell killing.

MATERIALS AND METHODS

Reagents. The double-stranded synthetic polyribonucleotide polyinosinic acid-polycytidylic acid (poly I:C) was obtained from Calbiochem, San Diego, CA. Bacterial lipopolysaccharide (LPS) was the kind gift of Dr. David Morrison, Emory University, Atlanta, GA. The immunoglobulin fraction of goat antiserum to mouse IFN α/β was provided by Dr. Donna Murasko of the Medical College of Pennsylvania, Philadelphia. One ml of anti- IFN α/β was capable of neutralizing approximately 2,000,000 units of IFN α/β . Rabbit antiserum to partially purified IFNγ was prepared in our laboratory as previously described (9). The immunoglobulin fraction was capable of neutralizing approximately 2,000 units of IFNγ.

Bone marrow-derived macrophages. Bone marrow cells were aspirated from the femura of 8 to 12 wk old C3H/HeN male mice and cultured at 37°C in 5% CO_2 in bone marrow medium consisting of H-MEM supplemented with 1% glutamine, 15% L-cell conditioned media, 10% FBS (low endotoxin fetal bovine serum) from HyClone Laboratories, Logan, Utah, and 5% horse serum. Pure macrophages were obtained after 12 to 14 days of culture and were used in priming/activation studies (10).

Priming/activation of macrophages for tumor cell killing. C3H/HeN mouse bone marrow macrophages were seeded at 5×10^5 cells in 0.1 ml volumes (in H-MEM-10% FBS) into flat-bottom 96-well tissue culture plates (CoStar). Killing mediated by the bone marrow macrophages was measured by a 16-hr ^{51}Cr release assay (11). In general, macrophages were preincubated with 0.1 ml of the indicated activators for 4 hr prior to addition of ^{51}Cr labeled P815 plasmacytoma cells.

IFN assay. IFN was assayed on mouse L cells using plaque reduction of vesicular stomatitis virus as the indicator virus as previously described (12). One unit of IFN caused a 50 percent reduction in plaque formation.

RESULTS AND DISCUSSION

Data are presented in Figure 1 on the activation of bone marrow macrophages by various concentrations of LPS in the absence of added IFN, and on the effect of antibodies to IFN α/β and IFNγ on this activation. Maximum activation of macrophages for P815 tumor cell killing occurred with $\geq$ 3 ng/ml LPS. Antibodies to IFN α/β blocked activation of the macrophages, while anti-IFNγ antibodies had no effect. The data suggest that LPS activated the macrophages for tumor cell killing by first inducing IFN α/β production by the macrophages. The produced IFN α/β , acting in an autocrine or paracrine

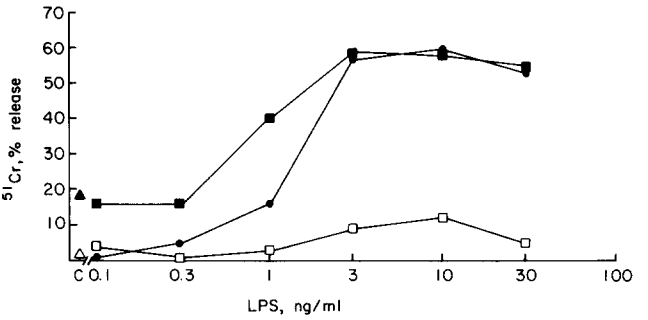

Figure 1. LPS activation of C3H/HeN mouse bone marrow macrophages for tumor cell killing. Anti-IFN α/β antibody was used at a final concentration sufficient to neutralize at least 30,000 units of IFN α/β , while anti-IFNγ was used at a final concentration sufficient to neutralize at least 1,000 units of IFNγ . Cytotoxicity was measured as ^{51}Cr released from prelabeled P815 mastocytoma cells after 16 hr incubation. Symbols: (●), LPS; (□), LPS + anti-IFN α/β ; (■), LPS + anti-IFNγ ; (△), anti-IFN α/β ; (▲), anti-IFNγ .

manner (13), provided the priming signal. LPS, in addition to induction of IFN α/β , also provided the triggering signal for macrophage killing. As previously demonstrated (14), treatment of macrophages with various concentrations of IFN α/β in the absence of LPS did not result in activation (data not shown). Thus, direct activation of the bone marrow macrophages by LPS really involved the two-signal priming/activation that has previously been described (1-3). Furthermore, variations in sensitivity by different macrophage preparations to direct activation by LPS may reflect differences in the ability of LPS to induce IFN in such cultures (11).

Poly I:C, an inducer of IFN α/β (Reviewed in 15), has also been shown to activate macrophages to kill tumor cells (4,7,8). It has been suggested that this activation is not a two-signal event, since it occurs in the absence of added macrophage activation factor (IFN?) (8). Bone marrow macrophages were treated with various concentrations of poly I:C in the presence of antibodies to IFN α/β and IFNγ in order to determine if IFN was also involved in poly I:C activation of macrophages (Figure 2). Significant activation of macrophages for killing P815 targets occurred with 10 ug/ml or more of poly I:C, which is similar to previous dose-response activation titrations of the polyribonucleotide (4,8). Activation was significantly blocked by antibodies to IFN α/β , but was not affected by anti-IFNγ antibodies. Thus, poly I:C was

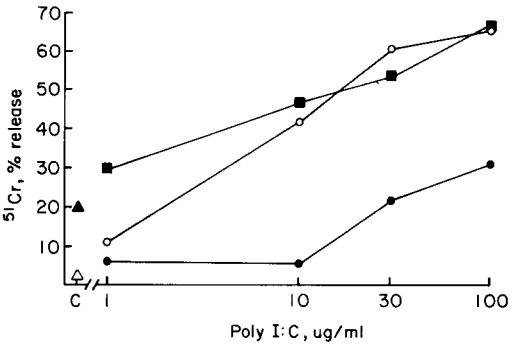

Figure 2. Poly I:C activation of C3H/HeN mouse bone marrow macrophages for tumor cell killing. Culture conditions were the same as described in Figure 1. Symbols: (○), poly I:C; (●), poly I:C + anti-IFN α/β ; (■), poly I:C + anti-IFN γ; (△), anti-IFN α/β ; (▲), anti-IFNγ .

similar to LPS in activation of macrophages for tumor cell killing in that the activation was blocked by anti-IFN α/β antibodies, and therefore probably involved induced IFN α/β as the priming signal.

It is unlikely that contamination by LPS or endotoxin was responsible for the poly I:C effects, since 10 ug of the poly I:C contained less than 0.005 ng LPS by the Limulus amebocyte lysate assay based on a negative test at 100 ug poly I:C (14). The data in Figure 1 demonstrate that the contamination would have had to be at least 1 ng or more of LPS per 10 ug poly I:C in order for LPS to have induced the activation of the macrophages. Thus, the activation presented in Figure 2 was due to poly I:C.

Both LPS and poly I:C are known inducers IFN α/β (15,16). Their induction of IFN α/β in the bone marrow cultures is illustrated in Table 1, where the induced activity was specifically sequestered and not detected in cultures containing anti-IFN α/β antibodies during the stimulation period. Concentrations of LPS or poly I:C that did not activate macrophages also failed to induce IFN. It has been shown that several hundred units/ml of IFN α/β are required to induce priming under conditions where IFN α/β is added to macrophage cultures (14). Although the concentrations of IFN α/β induced in macrophage cultures by LPS and poly I:C were $\geq$ ten-fold less than that reported to be required for priming, it is quite likely that the IFN α/β concentrations

Table 1

IFN production by bone marrow macrophages treated with LPS or poly I:C[a]

Inducer	Media	IFN activity in presence of (U/ml ± SD): Anti-IFN α/β	Anti-IFNγ
LPS (30 ng/ml)	23 ± 4	< 3	20 ± 14
LPS (3 ng/ml)	< 3	< 3	< 3
Poly I:C (30 ug/ml)	30 ± 0	< 3	30 ± 0
None	< 3	< 3	<3

[a]Anti-IFN α/β was used at a final concentration sufficient to neutralize 30,000 units of IFN α/β and anti-IFNγ was used at a final concentration sufficient to neutralize 1,000 units of IFNγ.

were actually much higher in the microenvironment of the primed macrophage since the responding cell is also the producer of the IFN. Thus, the concentrations of IFN α/β functioning in an autocrine and paracrine manner were probably much higher than those observed at the level of the culture as a whole.

In order to examine the specific triggering properties of poly I:C, macrophage cultures were primed with various concentrations of IFNγ in the presence of poly I:C and antibodies to IFN α/β . As shown in Figure 3, 10 ug/ml of poly I:C were able to trigger macrophage killing in proportion to the concentration of added IFNγ in the presence of anti-IFN α/β antibodies.

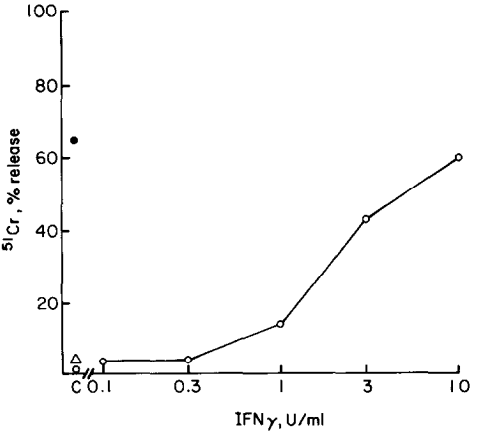

Figure 3. Poly I:C triggering of bone marrow macrophages that were primed by IFNγ in the presence of anti-IFN α/β (neutralizes 30,000 units of IFN α/β). Culture conditions were the same as described in Figure 1. Symbols: (○), poly I:C (10 ug/ml) + anti-IFN α/β ; (●), poly I:C (10 ug/ml); (△), IFNγ (10 units/ml).

Higher concentrations of poly I:C shifted the curve to the left, while lower concentrations shifted it to the right, thus demonstrating dose-response characteristics of LPS triggering (11). The data clearly show that poly I:C possesses macrophage triggering properties that are independent of its induction of IFN α/β in the cultures.

The data on poly I:C triggering suggest new and interesting approaches to studying the mechanism of triggering macrophages for tumor cell killing. Current evidence suggests that double-stranded RNAs (dsRNAs) such as poly I:C exert their effects on cells (IFN production, for example) by acting intracellularly (Reviewed in 15). Single-stranded RNAs (ssRNAs) that can form double-stranded regions can also carry out functions associated with dsRNAs (17). An IFN induced protein kinase that regulates protein synthesis requires dsRNA for activity (18). Reovirus ssRNA can replace the dsRNA requirement because it possesses significant double-strandedness in its structure (17). Interestingly, c-myc mRNA also has the ability to form dsRNA structures (19,20), and since LPS induces transcription of the c-myc gene (21), it is possible that ssRNA with dsRNA properties may mediate the LPS trigger. This model is not at odds with the recent reports of down-regulation of ribosomal RNA levels in activated macrophages (22,23), since the model deals with an mRNA with the property of double-strandedness and not with total RNA levels. Finally, since IFN down regulates RNAs such as c-myc mRNA (24,25), possibly via activation of an RNA endonuclease (18), it provides both a priming and regulatory signal for activation of macrophages in the context of dsRNA triggering.

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