DIFFERENTIAL EFFECTS OF POLYADENYLIC:POLYURIDYLIC ACID AND LIPOPOLYSACCHARIDE ON THE GENERATION OF CYTOTOXIC T LYMPHOCYTES *

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The generation of cytotoxic T lymphocytes (CTLs) in mixed leukocyte culture (MLC) reactions involves the interaction between different subpopulations of T cells (1). Precursors of CTLs (pre-CTL) respond primarily to antigens controlled by the H-2K and H-2D regions and develop into specific CTLs; these CTLs recognize the sensitizing H-2K and H-2D region-associated cytotoxic determinant (CD) antigens on target cells in the cell-mediated lympholysis (CML) assay (1-3). Another population of T cells responds to H-2I region-associated lymphocyte-defined determinant(s) (LD) with active proliferation and provides a helper effect in the development of CTLs (1-4). Thus, when responding and stimulating cells differ for both H-2I and H-2K or H-2D regions, there are strong proliferative and cytotoxic responses. If they differ only for the H-2K or the H-2D region, a low proliferative response and a weaker cytotoxic response are generated presumably due to weak LD-like activity associated with the H-2K and H-2D regions (5). When ultraviolet (UV) light-treated stimulating cells are used, there is no measurable proliferative or cytotoxic response even though the UV light-treated stimulating cells have the capacity to present the CD antigen. This is thought to be the result of the abolition of LD-like activity of the stimulating cells by UV light treatment and the lack of helper effect in the absence of the LD stimulus (6).

One approach to a study of the mechanisms underlying cell interactions involved in the generation of CTLs is to use agents that can modify or substitute for the helper effect. We now report the finding that the double stranded RNA, polyadenylic acid (poly A):polyuridylic acid (poly U) (poly A:U) and bacterial lipopolysaccharide W Escherichia coli 0127:B8 (LPS) can augment significantly the generation of CTLs in responder-stimulator cell combinations which differ for either the H-2K or the H-2D region. Two lines of evidence suggest that these two agents exert their effect at different steps in the development of the

* Supported by National Institutes of Health grants CA-16836 and AI-08439. This is paper no. 139 from the Immunobiology Research Center, the University of Wisconsin, Madison, Wis., 53706.

Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; BAEE, benzoyl-L-arginine ethyl ester; CD, cytotoxic determinant; CML, cell-mediated lympholysis; CRBC, chicken red blood cells; CTL, cytotoxic T lymphocytes; IgG, immunoglobulin G; LD, lymphocyte-defined determinant; LPS, lipopolysaccharide W Escherichia coli 0127:B8; MLC, mixed leukocyte culture; poly A:U, polyadenylic acid:polyuridylic acid; UV, ultraviolet.

J. Exp. Med. © The Rockefeller University Press - 0022-1007/78/0501-1355$1.00 1355
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cytotoxic response: (a) the effect of poly A:U depends on the presence of adherent cells, whereas the effect of LPS is independent of the presence of adherent cells, and (b) LPS promotes the development of CTLs when UV light-treated stimulating cells are used in the MLC whereas poly A:U does not.

Materials and Methods

Mice. The following mouse strains were used in this study. The letters in parentheses refer to the genotype derivation of the different regions of H-2, including K, I, S, and D: AQR (kkdd), B10.A (kkdd), B10.T(6R) (qqqd), B10.A(1R) (kkdb), B10.S (ssss), and C57BL/10 (bbbb).

Antiserum. BALB/c hyperimmune antiserum against chicken red blood cells (CRBC; ?) was a gift from Dr. Sylvia B. Pollack, Fred Hutchinson Cancer Research Center, Seattle, Wash.

In Vitro Sensitization and CML Assay. Generation of cytotoxic effector lymphocytes and CML assays were done according to the method of Peck and Bach (8). Stimulating cells were either mitomycin C-treated (indicated by the subscript m) or X-irradiated (indicated by the subscript x). The cells were cultured for 5 days and then the cytotoxicity determined by the CML assay. Percent cytotoxicity is expressed as the mean of four determinations ± SD.

Treatment with Poly A:U and LPS. Poly A:U (Miles Laboratories, Inc., Elkhart, Ind.) and LPS (lipopolysaccharide W Escherichia coli 0127:B8 obtained from Difco Laboratories, Detroit, Mich.) were added at the concentrations of 20 and 5 μg/ml, respectively, at the start of the culture.

UV Light Treatment of the Sensitizing Cells. To eliminate weak LD-like differences associated with H-2K and H-2D regions, sensitizing cells were UV irradiated according to the method of Lafferty et al. (9) with modification as described by Kuperman and Bach (10).

Antibody-Dependent Cellular Cytotoxicity (ADCC) Assay. The ADCC assay was carried out essentially as described by Pollack (11). Except where mentioned otherwise, the assay was done in Linbro microtitre plates (Linbro Chemical Co., Hamden, Conn.) with 2 × 10⁴ antibody-coated CRBC targets per well in a final volume of 0.2 ml.

Trypsin Treatment of Effector Cells. Effector cells from MLCs and normal spleen cells were treated with 0.25% trypsin (twice crystallized, 10,800 benzoyl-L-arginine ethyl ester (BAEE) U/mg, obtained from Grand Island Biological Co., Grand Island, N.Y.) in Hank's balanced salt solution containing NaHCO₃ for 45 min at 37°C, and afterwards the cells were washed twice.

Preparation of Aggregated Immunoglobulin G. Human immunoglobulin G (IgG), obtained from Miles Laboratories, was heat-aggregated by incubation at 63°C for 15 min at a concentration of 20 mg/ml in phosphate-buffered saline, pH 7.4.

Results

Enhancement of CTL Generation by Poly A:U and LPS. We have observed that both poly A:U and LPS enhance the generation of CTLs in several responder-stimulator combinations which differ for both the H-2I and the H-2K or H-2D regions or for the entire H-2 complex (data not shown). In such combinations there is generally a strong proliferative response and marked generation of CTLs even in the absence of poly A:U and LPS. In contrast, in combinations that differ for the H-2K or H-2D region only, both responses are generally very low.

Table I shows the effect of poly A:U and LPS on the generation of CTLs in such combinations using the responder-stimulator pairs AQR-B10.A and B10.A(1R)-B10.A which differ for H-2K and H-2D, respectively. The presence of LPS or poly A:U in the culture enhances the generation of CTLs in both the combinations. The optimum concentrations are 5 μg/ml for LPS and 20 μg/ml for poly A:U. LPS and poly A:U cause enhancement in the AQR-B10.A combination to a similar degree consistently, although the magnitude of the enhancement varies among experiments. In the B10.A(1R)-B10.A combination,
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TABLE I
Effect of Poly A:U and LPS in the Generation of CTLs in Responder-Stimulator Combinations Differing for H-2K Region and H-2D Region

| Responder + stimulator combination | Additions | Cytotoxicity ± SD on the targets |
|-----------------------------------|-----------|-------------------------------|
|                                   |           | B10.A (kkdd) | AQR (qkdd) | C57BL/10 (bbbb) |
| I AQR + B10.Am                    | None      | 4.8 ± 3.1    | 4.3 ± 3.0   | —               |
| AQR + B10.Am                      | LPS, 5 μg/ml | 30.7 ± 4.2    | 4.1 ± 3.6   | —               |
| AQR + B10.Am                      | Poly A:U, 20 μg/ml | 34.2 ± 8.5    | 6.4 ± 3.4   | —               |
| II B10.A(1R) + B10.A(1R),       | None      | −1.9 ± 7.6    | —           | —               |
| B10.A(1R) + B10.A(1R)           | LPS, 5 μg/ml | −5.0 ± 7.8    | —           | —               |
| B10.A(1R) + B10.A(1R)           | Poly A:U, 20 μg/ml | 2.0 ± 10.0    | —           | —               |
| III B10.A(1R) + B10.Ax          | None      | 11.6 ± 5.0    | —           | —               |
| B10.A(1R) + B10.Ax             | LPS, 5 μg/ml | 36.8 ± 9.4    | —           | —               |
| B10.A(1R) + B10.Ax             | Poly A:U, 20 μg/ml | 40.8 ± 3.0    | —           | —               |
|                                |           | 15.0 ± 6.8    | —           | 1.7 ± 5.7       |
|                                |           | 44.2 ± 2.6    | —           | −8.9 ± 9.6      |

Effector:target ratios were 100:1 in I, 50:1 in II, and 40:1 in III.

Both LPS and poly A:U caused similar enhancement in some experiments, but in others LPS caused enhancement to a greater degree than poly A:U.

The results shown in Table I were obtained at a single effector:target ratio for each experiment. However, the cytotoxic responses generated both in the presence and absence of LPS and poly A:U were measured at different effector:target ratios, and in all cases percent cytotoxicity was related linearly to the logarithm of effector:target ratio (data not shown) characteristic of T cell-mediated cytotoxicity (12).

The enhanced cytotoxicity observed is the result of enhanced generation of CTLs and not of the presence of poly A:U or LPS during the cytotoxicity assay as the addition of these agents during the CML assay does not affect cytotoxicity (data not shown). The CTLs generated in the presence of LPS and poly A:U are specific for the sensitizing antigen. In the B10.A(1R)-B10.A combination, the CTLs generated in the presence of poly A:U are cytotoxic for B10.A, whereas they are not or only minimally cytotoxic for C57BL/6 (bbbb) which does not share the sensitizing antigen, H-2D<sup>d</sup> (Table I). The CTLs are cytotoxic for B10.T(6R) (qqqd) which shares the sensitizing antigen and not cytotoxic for another nonspecific target B10.S (ssss) (results not shown). Similar results were also obtained with the CTLs generated in the presence of LPS. Furthermore, specific CTLs are also generated in the AQR-B10.A combination in the presence of these agents (data not shown).

Because LPS and poly A:U are known to act as adjuvants in antibody response (13-16), experiments were carried out to test whether there was any contribution by ADCC to the enhancement of cytotoxic response observed. AQR spleen cells were stimulated with X-irradiated B10.A spleen cells in the
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**Table II**

*Effect of Treatment with Trypsin or Aggregated IgG on the Cytotoxic Cells*

| Effector cells | Treatment | Effector: target ratio | Cytotoxicity ± SD (CML assay using B10.A CRBC targets) | ADCC assay using CRBC targets |
|----------------|-----------|------------------------|------------------------------------------------------|-----------------------------|
| AQR + B10.Ax   | None      | 50:1                   | 25.6 ± 2.8                                           | 11.6 ± 3.9                  |
| AQR + B10.Ax   | Trypsin   | 50:1                   | -5.0 ± 1.9                                           | -                           |
| AQR + B10.Ax   | Aggregated IgG | 50:1                 | 30.1 ± 2.2                                           | -                           |
| AQR + B10.Ax + LPS | None    | 50:1                   | 78.0 ± 3.9                                           | 5.4 ± 3.5                   |
| AQR + B10.Ax + LPS | Trypsin | 50:1                   | 7.6 ± 8.5                                            | -                           |
| AQR + B10.Ax + LPS | Aggregated IgG | 50:1                 | 63.5 ± 17.2                                          | -                           |
| AQR + B10.Ax + poly A:U | None    | 50:1                   | 71.6 ± 9.8                                           | 6.7 ± 1.2                   |
| AQR + B10.Ax + poly A:U | Trypsin | 50:1                   | 25.1 ± 8.3                                           | -                           |
| AQR + B10.Ax + poly A:U | Aggregated IgG | 50:1                 | 72.3 ± 9.7                                           | -                           |
| Normal AQR spleen cells | None    | 50:1                   | - 55.0 ± 8.0                                         | -                           |
| Normal AQR spleen cells | 12.5:1  | -                      | 28.4 ± 4.6                                           | -                           |
| Normal AQR spleen cells | Trypsin | 12.5:1                 | 28.9 ± 4.0                                           | -                           |
| Normal AQR spleen cells | Aggregated IgG | 50:1                 | 7.8 ± 4.0                                            | -                           |

Effector cells were generated in MLC in the presence and absence of LPS or poly A:U as indicated except in the case of normal spleen cells. Aggregated IgG was added to the assay mixture at a concentration of 4 mg/ml. 1:5 dilution of the anti-CRBC antiserum was used in the ADCC assay.

Presence and absence of LPS or poly A:U, and with the resulting cells CML assay using B10.A target cells and ADCC assay using CRBC-anti-CRBC system were carried out. As shown in Table II both LPS and poly A:U enhanced the cytotoxicity in CML but did not enhance the cytotoxicity in ADCC. The effector cell in ADCC is trypsin resistant (17, 18), whereas the cytotoxic T lymphocyte is sensitive to trypsin treatment (18). The data presented in Table II show that the cytotoxic effector cells generated in the presence of LPS and poly A:U are sensitive to trypsin treatment. Under the same experimental conditions, normal spleen cell population which participates in ADCC is trypsin resistant (Table II). Furthermore, aggregated IgG, which is known to inhibit ADCC (18), does not inhibit the cytotoxicity by the cells generated in cultures containing LPS or poly A:U. All these results clearly indicate that there is no measurable contribution by ADCC to the cytotoxic responses generated in the presence of LPS and poly A:U.

Table III shows the effect of poly A:U and LPS on the generation of CTL in the presence and absence of adherent cells. In the absence of adherent cells poly A:U does not enhance the generation of CTLs. LPS, on the other hand, enhances CTL generation both in the presence and absence of adherent cells.

In the two responder-stimulator combinations described above, there is a weak proliferative response and a weak cytotoxic response when X-irradiated stimulating cells are used. This is thought to be due to weak "LD-like" differences associated with the H-2K and H-2D regions as described elsewhere (5). The weak LD stimulus and the weak proliferative response can be abolished by using UV light-treated stimulating cells which do, however, present CD
TABLE III
Effect of Adherent Cell Removal on the Action of Poly A:U and LPS

| Responder + stimulator combination | Additions       | Cytotoxicity ± SD on B10.A target cells |
|-----------------------------------|----------------|-----------------------------------------|
|                                   |                | + Adherent cells | − Adherent cells |
| B10.A(1R) + B10.Ax                | None           | 9.3 ± 4.3        | 1.3 ± 2.6        |
| B10.A(1R) + B10.Ax                | Poly A:U, 20 μg/ml | 33.1 ± 1.8      | 3.0 ± 2.6        |
| B10.A(1R) + B10.Ax                | LPS, 5 μg/ml   | 61.9 ± 3.0       | 55.9 ± 3.9       |

Spleen cells, 30 × 10⁶ in 4 ml of medium were taken in 60 × 15-mm Falcon tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and incubated for 90 min at 37°C in an atmosphere of 95% air and 5% CO₂. The nonadherent cells were transferred to a fresh dish, and the incubation was continued for an additional 90 min. The nonadherent cells were harvested and used in the MLC reaction. Nonadherent responder and stimulator cells were prepared separately. Effector:target ratio was 40:1.

TABLE IV
Effect of Poly A:U and LPS in the Presence of UV Light-Treated Stimulating Cells

| Responder + stimulator combination | Additions       | Cytotoxicity ± SD on the targets |
|-----------------------------------|----------------|----------------------------------|
|                                   |                | B10.A (kkdd) | B10.T (6R) (qqqd) |
|                                   |                | %               | %               |
| AQR + AQRx                        | None           | −4.7 ± 2.0      | −3.1 ± 2.9      |
| AQR + B10.Ax                      | None           | 5.6 ± 1.0       | −1.8 ± 1.6      |
| AQR + B10.Ax                      | None           | −8.5 ± 3.0      | −2.3 ± 3.0      |
| AQR + B10.Ax                      | Poly A:U, 20 μg/ml | −3.6 ± 3.8     | −1.7 ± 1.5      |
| AQR + B10.Ax                      | LPS, 5 μg/ml   | 23.3 ± 3.1      | −2.3 ± 1.5      |

Effector:target ratio in the cytotoxicity assay was 70:1.

antigens to the responding cells (6). The effects of poly A:U and LPS on the generation of CTLs in the absence of the weak LD-like stimulus was thus tested using UV light-treated stimulating cells. As shown in Table IV poly A:U does not enhance the generation of CTLs whereas LPS retains the enhancing effect when UV light-treated stimulating cells are used.

Discussion

Our observation that both LPS and poly A:U enhance the generation of CTLs in responder-stimulator combinations that differ for both the H-2I and the H-2K or H-2D regions or for the entire H-2 complex is similar to the finding of Wagner and Cone (19). These investigators found that poly A:U amplified the generation of CTLs in in vitro response of CBA (H-2k) cells against mitomycin-treated BALB/c (H-2d) cells; they did not test the effect of LPS in their system. In these responder-stimulator combinations there is generally a strong proliferative response and marked generation of CTLs even in the absence of LPS and
poly A:U. In contrast, a responder-stimulator combination that differs for the H-2K or H-2D region shows low level proliferative and cytotoxic responses (5). In such combinations both poly A:U and LPS enhance the generation of cytotoxic cells significantly. This augmentation in cytotoxicity is due to an increased generation of CTLs and not to increased ADCC (Table II). The CTLs generated in the presence of these agents are specific for the sensitizing antigen.

In the presence of poly A:U, the incorporation of $[^3H]thymidine$ by the cells in the MLC is enhanced two- to threefold. LPS, on the other hand, enhances $[^3H]thymidine$ incorporation 8- to 10-fold (results not shown). It is not clear whether this effect on proliferative response has a role in the enhanced generation of CTL.

When UV light-treated stimulating cells are used, neither the proliferative response nor CTL generation are detectable, as previously described (6), presumably due to abolition of LD-like activity on the stimulating cells by UV light irradiation and the resulting lack of initiation of any response. In the presence of LPS, however, a cytotoxic response is generated against the CD antigens on the UV light-treated stimulating cells. On the other hand, no cytotoxic response is generated against UV light-treated stimulating cells in the presence of poly A:U. These results are consistent with the notion that LPS but not poly A:U has the capacity to exert appropriate signals and promote the initiation of cytotoxic response in the presence of UV light-treated stimulating cells and in the absence of any LD or LD-like activity. An alternate explanation is that LPS acts on UV light-treated stimulating cells and allows the expression of LD-like activity. But this possibility is less likely as UV light treatment has a deleterious effect on the metabolic activity of the cells.

Both poly A:U and LPS act as adjuvants in antibody response to T-dependent antigens (13-16), and they are thought to exert the adjuvant effect through helper T cells (20-22). It is conceivable that the enhancement of CTL generation caused by LPS and poly A:U in an MLC is also due to their effect on helper T cells.

The present results suggest that LPS and poly A:U enhance cytotoxic responses by different mechanisms. Poly A:U may only be able to amplify an ongoing cytotoxic response, whereas LPS may have the capacity to promote the initiation of cytotoxic response and also amplify the response. Because both the agents are potent at expanding an ongoing response, the inability of poly A:U to help initiate a response suggests that the signals for initiation and expansion of the cytotoxic response may be different. That these two agents exert their effect at two different steps in the development of CTLs is also supported by the finding that poly A:U requires the presence of adherent cells for its action whereas LPS does not under the in vitro conditions used.

Summary

In a mixed leukocyte culture (MLC) reaction of allogenic mouse spleen cells differing for H-2K or H-2D, only a weak cytotoxic response is generated. This cytotoxic response is augmented significantly if bacterial lipopolysaccharide (LPS), 5 μg/ml, or polyadenylic acid (poly A):polyyuridylic acid (poly U), 20 μg/ml, is present in the culture. The cytotoxic cells generated in the presence of
these two agents are specific for the sensitizing H-2K or H-2D antigen. Two lines of evidence suggest that these two agents exert their effect at different steps in the development of cytotoxic lymphocytes: (a) the effect of poly A:U depends on the presence of adherent cells, whereas the effect of LPS is independent of the presence of adherent cells and (b) LPS promotes the development of cytotoxic cells when ultraviolet light-treated stimulating cells are used in the MLC whereas poly A:U does not.

We thank Dr. Sylvia B. Pollack, Fred Hutchinson Cancer Research Center, Seattle, Wash., for her generous gift of anti-CRBC antiserum. We also thank Prof. Fitz H. Bach for his support, encouragement, and help in the preparation of this manuscript.

Received for publication 6 September 1978.

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