THE INDUCTION OF VIRUS-SPECIFIC CYTOTOXIC T LYMPHOCYTES WITH SOLUBILIZED VICAL AND MEMBRANE PROTEINS*

By ROBERT FINBERG, MATTHEW MESCHER, AND STEVEN J. BURAKOFF

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Murine cytotoxic T lymphocytes (CTL)\(^1\) generated in response to virally infected syngeneic cells have specificity for both viral and H-2K and/or D antigens (1). In the case of Sendai virus, viral infection is not required and CTL can be induced with virus inactivated by ultraviolet light (2) or chemical treatment (3). These CTL will lyse syngeneic target cells coated with inactivated virus. Although it is now clear that both viral antigen and H-2 antigen are recognized by virus-specific CTL, the molecular nature of the antigenic determinants is not known.

A better understanding of the antigenic determinants required for the induction of a virus-specific syngeneic CTL response could be obtained if it were possible to isolate the viral and H-2 antigens in a soluble, immunogenic form. This would allow the isolation of the necessary molecules and the study of their interactions. Membrane proteins can be solubilized by detergent in a manner which retains their ability to stimulate a secondary allogeneic CTL response when added to in vitro cultures in soluble form (4) or in the form of reconstituted membranes\(^2\) (5). This stimulation is specific and is lost upon removal of the H-2 molecules from the solubilized membrane proteins (4). In this report we demonstrate that a similar approach can be used to study the antigenic requirements for induction of a syngeneic, Sendai virus-specific secondary CTL response. When solubilized Sendai virus proteins and cell membrane proteins containing H-2 antigens are introduced together into reconstituted membranes, these reconstituted membranes are able to induce virus-specific CTL.

Materials and Methods

Mice. 6-12-wk-old mice of the C57BL/6 (B6) (H-2\(^b\)) and DBA/2J (H-2\(^d\)) strains were obtained from The Jackson Laboratory (Bar Harbor, Maine). B6D2F\(_1\) (H-2\(^b/\text{d}\)) mice were purchased from Cumberland Farms (Clinton, Tenn.).

Tumors. P815 (H-2\(^d\)) mastocytoma was maintained in ascites (with weekly passage in serum-free medium) in DBA/2 female mice. EL-4 (H-2\(^a\)) leukemia was maintained in the same way in C57BL/6 mice.

Virus. Inactivated Sendai virus was obtained from Connaught Laboratories, lot 150-1, (Willowdale, Ontario). The virus had been propagated in pathogen-free chicken eggs and was inactivated by treatment with \(\beta\)-propiolactone. The hemagglutination titre was 1:4,096 per

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Abbreviations used in this paper: CTL, cytolytic T lymphocytes; DOC, deoxycholate; F, fusion; HN, hemagglutinin; M, matrix; NP, nucleoprotein; P, polymerase; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

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0.25 ml of virus suspension. The virus was stored at -70°C. Before use, cell debris was removed from the virus suspension by centrifugation at 1,500 rpm for 10 min.

**Generation of Effector Cells.** Mice were immunized by a 0.05-ml i.p. injection of the virus suspension. Spleen cells were removed 1-2 wk later and cultured in vitro with either intact stimulator cells coated with virus or with reconstituted membranes or liposomes prepared as described below. Cells were cultured in 2-ml culture wells (Linbro Chemical Co., New Haven, Conn.) using 7 × 10⁶ responder cells per well under the conditions previously described (6).

Intact, virus-coated stimulator cells were prepared by a modification of the procedure described by Schrader and Edelman (2). 50 × 10⁶ whole spleen cells, after Tris-NH₄Cl treatment and irradiation (1,500 rads, GE Maxiμr 250), were incubated with 0.01 ml of virus suspension for 90 min in 2 ml of culture medium and then washed twice to remove free virus particles. 6 × 10⁶ Sendai-coated stimulator cells were used per culture well.

**³¹Cr-Release Assay.** After 5 days of culture, cytotoxicity was assessed in a 4-h chromium release assay as previously described (6). Sendai virus-coated target cells were prepared by incubating 3 × 10⁶ Na⁺⁻⁻⁴CrO₄ labeled P815 or EL-4 tumor cells for 90 min with 0.01 ml of virus suspension at 37°C. Specific release is defined as E-C/FT-C (6).

**Tumor Cell Membranes.** Membranes were prepared from P815 tumor cells as previously described (7) but the final density gradient centrifugation to separate plasma membrane from endoplasmic reticulum was omitted. Briefly, cells were lysed by nitrogen cavitation, nuclei and mitochondria were removed by low speed centrifugation, and a particulate fraction was obtained by centrifugation at 20,000 g for 30 min. This membrane preparation, containing plasma membrane and endoplasmic reticulum, was then washed with 0.01 mM Tris, pH 8, containing 0.14 M sodium chloride (Tris-buffered saline, TBS) and resuspended in the same buffer.

**Membrane and Virus Solubilization and Reconstitution.** Tumor cell membranes were solubilized in 0.5% Na deoxycholate (DOC; Sigma Chemical Co., St. Louis, Mo.) as previously described (4) except that the buffer used was TBS. Sendai virus proteins were solubilized by suspending TBS-washed viral particles in TBS containing 0.5% DOC at a detergent to protein ratio (wt/wt) of 10:1. Each suspension was incubated for 15 min at 4°C and centrifuged for 45 min at 100,000 g to remove detergent insoluble material. Approximately 65% of the viral protein and 75–80% of the P815 protein was solubilized under these conditions.

Three types of reconstituted vesicles were prepared as outlined in Fig. 1. Solubilized Sendai virus protein, solubilized tumor cell membranes, or a mixture of solubilized Sendai virus and tumor cell membranes were dialyzed in a 1,000-fold excess of TBS containing 5 mM CaCl₂. Dialysis was done at protein concentrations of 0.1–0.2 mg/ml. In some experiments, lipids extracted from P815 membranes were added to solubilized Sendai proteins before dialysis. After dialysis for 24 h at 4°C, samples were centrifuged at 100,000 g for 45 min to pellet the reconstituted membranes. The pellets were resuspended in a small volume of TBS, 5 mM CaCl₂ and placed into culture with responder cells. Reconstituted membranes were always used on the day they were prepared.

Approximately 44% of the solubilized P815 membrane protein was found in the reconstituted membranes and 56% of the solubilized Sendai protein. Addition of P815 membrane lipids (see below) before dialysis did not result in significantly greater incorporation of Sendai protein into reconstituted membranes. When solubilized P815 membrane proteins were mixed with Sendai proteins before dialysis (Fig. 1) the total amount of protein incorporated into reconstituted membranes indicated that the percent incorporation of membrane and virus proteins remained the same.

**Liposomes Containing Sendai Virus Proteins and Lectin Purified H-2 Antigen.** H-2 antigens from solubilized P815 membranes were partially purified by affinity chromatography on a *Lens culinaris* (lentil) lectin column (8). This results in 20-fold purification and the H-2 antigens retain activity for stimulation of an allogeneic CTL response. Lipids were extracted from P815 membranes with chloroform: methanol (2:1, vol:vol), the extract was washed with water (0.3 vol) and dried under a stream of nitrogen. Dried lipids were dissolved in 0.5% DOC in TBS at a concentration of 1 μmol phosphate/ml.

Liposomes containing Sendai proteins or Sendai proteins and partially purified H-2 proteins were prepared by mixing the solubilized proteins with phospholipid in 0.5% DOC at concentrations of 50 μg/ml protein and 0.15 μmol lipid (based on phosphate)/ml. The mixtures
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FIG. 1. Preparative scheme for solubilization of P815 tumor cell membranes and Sendai virus particles and formation of reconstituted membranes.

Results

Sendai Virus Specific, H-2-Restricted CTL Induction. B6(H-2\textsuperscript{b}) mice, primed in vivo with inactivated Sendai virus and challenged in vitro with virus-coated syngeneic stimulator cells, lysed syngeneic tumor target cells coated with virus (Table I). Only minimal lysis of H-2\textsuperscript{d} virus-coated target cells was detected. Conversely, CTL from H-2\textsuperscript{d} mice lysed H-2\textsuperscript{d} but not H-2\textsuperscript{b} virus-coated targets. To provide specificity controls in the experiments using reconstituted membranes, B6D2F\textsubscript{1} (H-2\textsuperscript{b/d}) mice were used. When primed in vivo with inactivated virus and challenged in vitro with virus-coated F\textsubscript{1} stimulator cells, lysis of both H-2\textsuperscript{b} and H-2\textsuperscript{d} virus-coated targets was found. If stimulated with parental H-2\textsuperscript{b} Sendai-coated cells, the B6D2F\textsubscript{1} responder cells were able to lyse EL4 (H2\textsuperscript{b}) Sendai targets and if stimulated with H-2\textsuperscript{d} Sendai-coated cells, P815 (H2\textsuperscript{d}) Sendai-coated targets were lysed (Table I). Cytolytic activity was shown to be of T-cell origin by its elimination with Lyt antisera.

Stimulation by Reconstituted Membranes. Three different types of reconstituted membrane preparations were used to examine the ability of Sendai and P815 membrane proteins to stimulate a secondary CTL response after detergent solubilization. Reconstituted membranes containing only P815 membrane proteins (P), only Sendai virus

\footnote{Burakoff et al. Manuscript submitted for publication.}
Table 1

*Immune spleen cells were cultured with Sendai virus-coated stimulator cells and cytotoxicity assayed on virus-coated P815 and EL-4 cells as described in Materials and Methods. Effector/target ratio was 80/1.

† Spontaneous release for Sendai-coated EL-4 ranged from 20 to 35% and for P815 from 9 to 29% in a 4-h assay. Results are given as percent specific release of $^{31}$Cr.

§ Not tested.

| Responder       | Stimulator               | Target‡       |
|-----------------|--------------------------|---------------|
|                 |                          | P815-S (H-2^d) | EL-4 Sendai (H-2^b) |
| B6              | B6-Sendai (H-2^a)       | 15            | 74              |
| DBA/2           | DBA/2-Sendai (H-2^a)    | 84            | 19              |
| B6D2F1          | B6D2F1-Sendai (H-2^d)   | 86            | 84              |
| B6D2F1          | B6-Sendai (H-2^a)       | NT§           | 93              |
| B6D2F1          | DBA/2-Sendai (H-2^a)    | 95            | NT              |

* Immune spleen cells were cultured with Sendai virus-coated stimulator cells and cytotoxicity assayed on virus-coated P815 and EL-4 cells as described in Materials and Methods. Effector/target ratio was 80/1.

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§ Not tested.

proteins (S), or a mixture of both P815 and Sendai proteins (M) were prepared (Fig. 1). When reconstituted membranes having both virus proteins and P815 (H-2^a) membrane proteins (M) were added to cultures of immune B6D2F1 spleen cells, significant activity was found only on Sendai-coated P815 (H-2^a) target cells and not on Sendai-coated EL-4 (H-2^b) target cells (Fig. 2). No lysis was detected on target cells not coated with virus. Thus, the H-2 antigens of the reconstituted membrane, and not the H-2 antigens of the responder cells, appear to determine the specificity of
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Fig. 3. CTL stimulation by liposomes. Primed (B6D2)F1 mice were cultured with Sendai virus-coated whole cells from DBA/2 mice (D2-S) or with liposomes. Liposomes contained Sendai virus proteins only (S, 8 μg protein) or virus proteins together with lectin-purified membrane proteins (ML, 7 μg protein). CTL activity on Sendai-coated P815 targets is shown. No lysis was detected on Sendai-coated EL4 targets. Background lysis by responder cells cultured alone was 13% at an E/T ratio of 80/1, 5% at 27/1, and 1% at 9/1. This is a representative experiment of a total of three experiments.

The response. This would suggest that the Sendai proteins were presented in the context of the P815 membrane H-2 antigens and not in association with the H-2 antigens of the F1 macrophages (or other responder cells) present in the culture.

When equivalent amounts of P815 membrane proteins and Sendai proteins were added to cultures in the form of separately reconstituted membranes (S and P), minimal cytolytic activity was generated (Fig. 2). In fact, the CTL activity was only slightly above that seen when reconstituted P815 membranes (P) were added alone to the culture. These observations further demonstrate the role played by the H-2 antigens of the reconstituted membranes and demonstrate that under these conditions the viral and H-2 antigens must be present in the same membrane. Experiments were also done to rule out the possibility that the Sendai proteins are incorporated into the reconstituted membranes differently in the mixture (M) than when reconstituted alone (S) due to the larger amount of lipid present in the mixture. This was done by adding isolated P815 membrane lipids, in amounts (based on phosphate) equivalent to that present in the mixture, to the solubilized Sendai proteins before dialysis. Reconstituted membranes formed in this manner were also ineffective in stimulating a response when added to cultures along with reconstituted P815 membranes.

Reconstituted membranes containing Sendai protein alone (S) (with or without added P815 lipids) did not induce CTL activity within the concentration range examined in these experiments. At high concentrations, some CTL activity could be detected on both H-2^d and H-2^b targets coated with Sendai virus (data not shown). Under these conditions, viral proteins may interact with the H-2 antigens of the F1 cells and be presented in this manner.

Stimulation by Liposomes Containing Sendai Proteins and Partially Purified H-2 Antigens. Liposomes containing both solubilized Sendai proteins and lectin-purified H-2^d antigens also stimulated a virus-specific H-2-restricted CTL response (Fig. 3). As
Fig. 4. SDS polyacrylamide gel electrophoresis of Sendai virus and liposomes. (A) Intact Sendai virus (2.5 μg). (B) Virus protein insoluble in 0.5% DOC (1.1 μg). (C) Virus protein solubilized in 0.5% DOC (1.1 μg). (D) Liposomes formed with soluble virus protein (1.4 μg). (E) Liposomes formed with soluble virus protein and lectin purified H-2 antigen (1.2 μg).

Sendai virus proteins are identified by their mobility on SDS gels according to Orvell and Norrby (12).

was the case with reconstituted membranes, the specificity of the response was determined by the H-2 antigens present in the liposomes and liposomes containing only Sendai proteins gave no stimulation.

Use of the 20-fold purified H-2 antigens made it possible to compare, by polyacrylamide gel electrophoresis, the Sendai protein content of the liposomes formed with and without added H-2 antigens. Under the conditions used for formation of the mixed liposomes, the only major protein bands detectable on SDS gels are the Sendai proteins (Fig. 4e). The individual protein components of the lectin-purified fraction are present in low enough concentration to be seen, if at all, as only faintly staining bands. Although H-2 antigens are not detectable on SDS gels, their presence is demonstrated by the specificity of the virus-specific CTL response. This was further confirmed by demonstrating that these same liposomes will stimulate a secondary, H-2d specific allogeneic CTL response by cells from B6 (H-2d) mice previously immunized in vivo with P815 cells (data not shown).

Sendai virus contains five major proteins detectable on SDS gel electrophoresis (Fig. 4a). Treatment of intact virus with deoxycholate results in solubilization of four of these (Fig. 4c). The nucleoprotein (NP) remains largely insoluble (Fig. 4b). Incorporation of the solubilized protein into liposomes in the absence (Fig. 4d) or presence (4e) of lectin-purified H-2 antigens results in liposomes containing primarily the hemagglutinin (HN); fusion (F) and matrix (M) proteins. NP and the polymerase (P) are present in very small amounts. The faintly staining bands seen in the mixed liposomes (Fig. 4e) and not in whole virus are proteins present in the lectin-purified H-2 preparation. These results demonstrate that both types of liposomes (S and ML) used in these studies contain the same Sendai virus proteins and thus confirm that the difference in their effectiveness in stimulating a CTL response (Fig. 3) is due to the presence or absence of the lectin-purified proteins and not to a difference in Sendai protein content.
Discussion

The experiments described in this report demonstrate that membrane antigens from virus and tumor cells can be obtained in soluble form and subsequently recombined in reconstituted membranes which are able to stimulate a virus-specific H-2-restricted secondary CTL response. The specificity of the induced CTL is dependent upon the H-2 antigens present in the reconstituted membranes or liposomes, and is not determined by the H-2 of the \( F_1 \) responder cells. Under the conditions used, activity was obtained only if P815 membrane proteins and Sendai virus proteins were reconstituted together, suggesting that both the H-2 antigens and viral proteins must be present in the same membrane.

What is the evidence that the H-2 antigens of the solubilized tumor cell membranes are required for the CTL induction seen in these experiments? Several observations suggest that this is the case. First, the response was directed only at the H-2\( ^d \) antigens present in the reconstituted membranes and not at the H-2\( ^a \) antigens present on the \( F_1 \) responder cells. Secondly, we have previously shown that the activity of solubilized P815 plasma membranes for induction of a secondary allogeneic response could be eliminated by removal of the H-2 antigens with anti H-2\( ^d \) antiserum and Protein A Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) (4). Third, 20-fold purification of the H-2 antigens by lectin chromatography resulted in material which retained the ability to stimulate when combined with Sendai proteins in liposomes.

In all of the experiments reported here, stimulation with reconstituted membranes or liposomes resulted in less CTL activity than was obtained by stimulation with Sendai virus-coated whole cells. This is consistent with our earlier finding that purified tumor cell plasma membranes are less efficient than whole cells in stimulating a secondary allogeneic CTL response (7). It is possible that such results might be due to susceptibility of membranes and liposomes to degradation by proteolytic activities in the culture supernate or due to their removal from the culture by macrophages. The possibility must also be considered that membranes and liposomes, although clearly possessing the determinants necessary for specific stimulation of CTL precursors, may have lost some determinants normally involved in the generation of a maximal response to whole cells. It is not unlikely that the ratio of virus protein to H-2 antigen and the density of these proteins in the liposome might also affect their efficiency. We are currently studying the effect of varying these parameters.

Whether the methods employed here can be extended to the study of CTL responses against viruses other than Sendai remains to be determined. With many other viruses, e.g., ectromelia (13), target cell lysis can be obtained only after viral protein synthesis has been allowed to occur. If, in these cases, the antigens involved in CTL induction cannot be isolated directly from the virion it should be possible to obtain the relevant antigens in soluble form from the membranes of virally infected cells.

The ability to obtain virus and membrane proteins in soluble form and to combine them in a manner which results in activity for the induction of a virus-specific CTL response should make it possible to isolate the viral and host cell antigens necessary for T-cell recognition. The feasibility of this is indicated by the experiments using partially purified H-2 antigens obtained by lentil lectin chromatography. Using purified, or partially purified antigens it should then be possible to investigate the association, if any, between viral and membrane proteins, and their relevance to CTL recognition.
Reconstituted membranes were prepared from detergent solubilized P815 (H-2d) tumor cell membranes and solubilized Sendai virus protein. These reconstituted membranes stimulated a virus-specific H-2-restricted secondary CTL response. Stimulating activity was dependent upon the presence of both viral and P815 protein in the same membrane and was restricted to the H-2 specificity present in the reconstituted membrane. Liposomes prepared from solubilized Sendai virus proteins and partially purified H-2 alloantigen also had activity for CTL induction. The results demonstrate the feasibility of using detergent solubilized membrane proteins to study antigen recognition by virus-specific, H-2 restricted cytolytic T lymphocytes.

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