JOINT RECOGNITION BY CYTOTOXIC T CELLS OF INACTIVATED SENDAI VIRUS AND PRODUCTS OF THE MAJOR HISTOCOMPATIBILITY COMPLEX*

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The exceptional antigenicity of the major histocompatibility antigens suggests that they may be directly involved in the mechanisms of immunity. In accord with this idea, evidence has recently been obtained indicating that the H-2 antigens play a critical role in the lysis of virally infected or neoplastic cells by syngeneic cytotoxic T cells (1–8). Cytotoxic T cells specific for virally infected cells recognize not only virus-specific determinants, but also specific H-2 determinants on the target cell (1–5). Similar observations have also been made in systems in which cytotoxic T cells are generated against chemically modified isologous cells (9, 10) or against minor histocompatibility antigens (11, 12).

Two general hypotheses can account for the observations that cytotoxic T cells raised against virally infected cells recognize not only virus-specific antigens but also the H-2 antigens of the infected cell: (*a*) Cytotoxic T cells may have two separate sets of receptors, one for the H-2 antigens, the other for neoantigens resulting from viral infection. (*b*) The T cell may have only one receptor which recognizes a complex antigen specified by both the H-2 genes and the virus. Presently available data do not allow the rejection of either of these hypotheses.

Some versions of the two-receptor hypothesis have been ruled out, however, by demonstrations that the sharing of H-2 antigens between cytotoxic T cells and virally infected or hapten-modified target cells is neither necessary (13–15) nor sufficient (1, 9, 10, 16) for lysis to occur. Further evidence against the two-receptor hypothesis has come from experiments on competitive inhibition of the lysis of virally infected (16), or hapten-modified, ^{51}Cr-labeled target cells (9, 10). Competition by unlabeled cells required that they possess H-2 antigens in common with the ^{51}Cr-labeled target cells and also that they be previously modified by treatment with the appropriate hapten or virus.

While these results favor the one-receptor hypothesis, the nature of the antigenic determinant on the target cell that is recognized by such a receptor is still an outstanding problem. We have reported evidence suggesting that viral and H-2 determinants can exist in close association on the cell membrane (7, 17). The molecular mechanism of this association is unknown, and a number of complex possibilities still remain to be excluded. For example, infection of a cell with a replicating virus offers possibilities for the interaction of *H-2* and viral genes or their products at the level of synthesis during replication, transcrip-

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tion, or translation. Alternatively, postsynthetic association or modification of the proteins may occur.

In the present study, we distinguish between synthetic and postsynthetic interactions by investigating whether coating a cell with a nonreplicating virus can render it susceptible to lysis by cytotoxic T cells specific for the virus. We have found that specific cytotoxic T cells can lyse syngeneic target cells which have not been infected with active Sendai virus, but are merely coated with inactivated virus. In accord with previous results (1–5), the ability of specific cytotoxic cells to kill these virus-coated target cells was restricted by the H-2 type of the target cells. These findings suggest that the T cell can recognize an antigenic determinant formed by the physical association of H-2 antigens and preformed antigens of the noninfectious Sendai virus.

Materials and Methods

Virus. UV-inactivated Sendai virus was obtained from Microbiological Associates, Bethesda, Md. Infectious virus was grown in eggs

Immunization. DBA/2J, C57BL/6J, and B10.D2 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, and, where indicated, these mice were immunized with 0.05 ml of UV-inactivated Sendai virus, given intraperitoneally These animals were used as donors of spleen cells for tissue culture from 1 to 8 wk later

In Vitro Generation of Cytotoxicity. Each flask (Falcon 3012, stood on end) contained 20×10^6 viable nucleated spleen cells plus a mixture of inactivated Sendai virus (0.1 ml) and 3×10^6 cells from the same pool. The mixture of spleen cells and virus was held at 4°C for 30 min before it was added to the culture flask. The medium was RPMI-1640 with 5% fetal calf serum (Associated Biomedic Systems, Buffalo, N. Y.), 0.292 mg/ml additional glutamine, and 2-mercaptoethanol (5×10^{-5} M). Cultures were harvested after 5 days, and the cells were resuspended in 0.6 ml of medium.

Cytotoxic lymphocytes were generated against allogeneic cells by culturing 20×10^6 spleen cells (e g , from DBA/2 mice) with 10^7 mitomycin C-treated allogeneic spleen cells (e.g., from C57BL/6 mice) Cells were treated with mitomycin C (Sigma Chemical Co., St Louis, Mo.) by incubation at 37°C for 30 min with 40 μg of mitomycin C per ml of medium (18).

Cytotoxic Assay A modified ^{51}Cr-release assay was used as described elsewhere (19); the usual incubation time was 4 h. Target cells were the DBA/2 mastocytoma, P815, and the C57BL/6 lymphoma, EL4.BU. The appropriate target cells were coated with inactivated Sendai virus by mixing 1.25×10^6 ^{51}Cr-labeled tumor cells with 0.1 ml of inactivated Sendai virus and 0.1 ml of medium. After 30 min at 4°C, the cells were washed three times with cold balanced salt solution and resuspended in 5 ml of medium for use in the cytotoxic assay.

Percent specific lysis was calculated from the following formula:

$$\% \text{ specific lysis} = \frac{\text{experimental counts released} - \text{control counts released}}{\text{total releasable counts} - \text{control counts released}}.$$

Treatment with Anti-Thy-1.2 Serum and Complement A two-stage procedure using AKR anti-C3H, anti-Thy-1.2 serum (Litton Bionetics, Kensington, Md.) and guinea pig complement (Grand Island Biological Co., Grand Island, N Y) was performed as described elsewhere (8).

Kinetic Studies. Centrifuge tubes were used instead of the microtiter trays of the standard assay in order to facilitate centrifuging mixtures of cytotoxic cells and target cells and therefore eliminate the time required in the usual assay for the cells to settle The cells were kept at 4°C in all steps before incubation of the mixture at 37°C. After various incubation periods, the tubes were placed in a 43°C water bath, and 2 ml of balanced salt solution, previously warmed to 43°C, was added to prevent further cell-mediated lysis (19, 20). After 60 min at 43°C to allow the release of ^{51}Cr from lysed cells (19, 20), the tubes were centrifuged and an aliquot of the supernate taken for counting in a scintillation counter. Controls for minimal and maximal release of ^{51}Cr were included for each time-point. All assays were performed in duplicate, and lysis of both normal and Sendai virus-coated target cells was determined at each time-point

Labeling of Cells by Incorporation of Radioactive Amino Acids P815 cells (2.5×10^6) were

coated with UV-inactivated Sendai virus by the procedure described above and cultured in 1 ml of medium for 10-16 h. Untreated P815 cells (5×10^6) were cultured in parallel. Virus-coated or untreated P815 cells (2.5×10^6) were then resuspended in 1 ml of leucine-deficient medium with 50 μCi of [^{3}H]leucine (New England Nuclear, Boston, Mass.) or 1 ml of methionine-deficient medium with 100 μCi of [^{35}S]methionine (New England Nuclear) Untreated P815 cells (2.5×10^6) were also labeled with [^{3}H]leucine to allow comparison of the relative incorporation of [^{3}H]leucine and [^{35}S]methionine in these cells.

After 2 h the cells were washed three times with balanced salt solution and dissolved in 0.2 ml of 5% sodium dodecyl sulfate (SDS) [1] In some experiments, the initial incubation period was omitted from this procedure.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in Tris-glycine buffer with SDS (21). Disk gels were 10% polyacrylamide. High resolution slab gels consisted of a gradient of 5–20% polyacrylamide, stabilized with a gradient of 5–10% sucrose.

Autoradiography. Gels containing ^{35}S-labeled material were exposed to Kodak X-Omat XR-5 film for 5 h at −70°C. Gels containing [^{3}H]leucine-labeled material were dehydrated, treated with 2,5-diphenyloxazole (22), and exposed to Kodak X-Omat XR-5 film for 6 h at −70°C.

Results

In vitro Generation of T-Cell-Mediated Cytotoxicity against Cells Coated with Inactivated Sendai Virus. Spleen cells (20×10^6) from normal DBA/2 mice were cultured together with 3×10^6 DBA/2 spleen cells which had been exposed to UV-inactivated Sendai virus (0.1 ml) for 30 min at 4°C. After 5 days, the cultures were harvested and assayed for the presence of cytotoxic activity specific for Sendai virus. The target cells were syngeneic ^{51}Cr-labeled P815 cells which had been coated with UV-inactivated Sendai virus (P815-SV). Cells from these cultures lysed P815-SV cells to a greater extent than untreated P815 cells (Fig. 1), suggesting the presence of a cytotoxic response specific for Sendai virus.

If this cytotoxic activity were due to an immune response, the in vivo exposure of donor mice to Sendai virus should have resulted in immunological memory. To investigate this question, DBA/2 mice were injected intraperitoneally with 0.05 ml of UV-inactivated Sendai virus. 3–6 wk later these mice were used as the donors of spleen cells, which were cultured as before with SV-coated isologous cells. As shown in Fig. 1, in vivo immunization of the donor mice with Sendai virus resulted in a greatly enhanced in vitro cytotoxic response to SV-coated cells.

The killing of SV-coated target cells by cultures of cells from mice immunized with Sendai virus in vivo only occurred after Sendai virus had been added to the cultures (Fig. 2). This implies that the cytotoxic activity in these cultures was the result of a secondary immune response which was initiated in vitro, and not of the continuation in vitro of the in vivo primary response.

The nature of the cytotoxic cells was ascertained by treating the cultured cells before the cytotoxic assay with anti-Thy-1.2 serum and complement. Pretreatment with anti-Thy-1.2 serum and complement abolished the cytotoxic activity (Fig. 3), indicating that the cytotoxic activity depended on the presence of T cells.

Absence of Nonspecific Lysis of Sendai Virus-Coated Target Cells. Although the results of in vivo immunization of spleen cell donors (Figs. 1–3) indicated

[1] *Abbreviations used in this paper:* P815-SV, P815 cells coated with inactivated Sendai virus; SDS, sodium dodecyl sulfate.

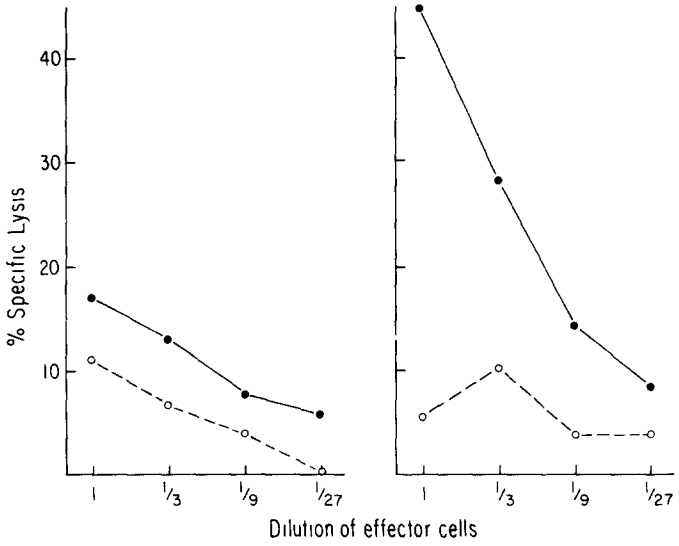

FIG. 1. Generation of cell-mediated cytotoxicity against Sendai virus-coated cells in cultures of lymphocytes from normal or immunized mice. Spleen cells from normal DBA/2 mice or mice immunized 3 wk before with 0.05 ml of inactivated Sendai virus were cultured for 5 days in the presence of syngeneic cells, coated with inactivated Sendai virus as described in the text. Cultures were harvested, the cells resuspended in 0 6 ml of medium, and cytotoxic activity assayed against ^{51}Cr-labeled normal P815 cells (--O--) or P815-SV (—●—) The graph on the left shows the specific lysis of these target cells by cultures of normal spleen cells; the graph on the right shows the specific lysis by cultures of cells from immunized mice

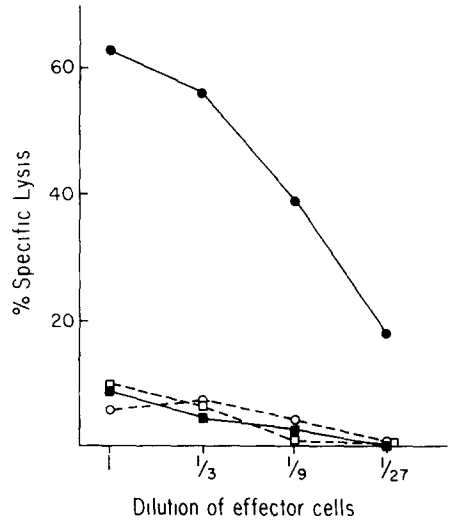

FIG 2. Requirement for addition of inactivated Sendai virus in vitro in order to generate cytotoxic activity. Spleen cells from DBA/2 mice previously immunized with inactivated Sendai virus were cultured with DBA/2 cells that had been incubated with inactivated Sendai virus as described above (●, ○), or with DBA/2 cells incubated with medium alone (■, □) Cultures were assayed against normal P815 cells (----) or P815-SV (——)

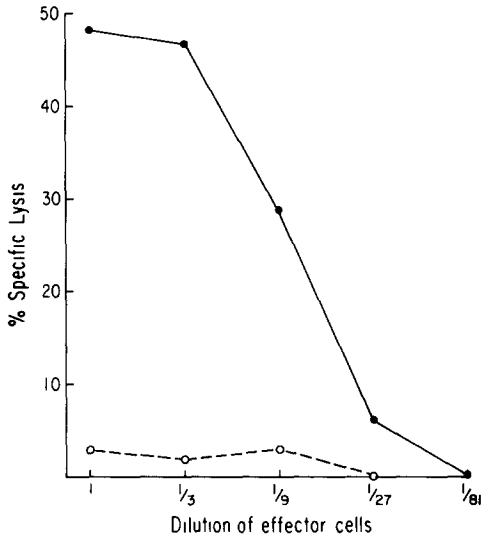

FIG. 3 T-cell dependence of the cytotoxic activity Spleen cells from immunized mice were cultured as before with inactivated Sendai virus Before assay, one aliquot of the cells was treated with anti-Thy-1.2 serum and complement, while a control aliquot was treated with normal AKR serum and complement Shown is the specific lysis of P815-SV by the aliquot treated with anti-Thy-1.2 serum (--○--) and by the aliquot treated with normal AKR serum (—●—) The specific lysis of normal P815 target cells by this population of cytotoxic cells was less than 5%.

that specific cytotoxic T cells were being generated against antigens specified by the Sendai virus, it was still possible that the target cells coated by Sendai virus were lysed in a nonspecific way. For example, the Sendai virus, which can agglutinate lymphoid cells (23), could be binding together cytotoxic lymphocytes and Sendai virus-coated target cells irrespective of the immunological specificity of the cytotoxic lymphocytes. This agglutination could result in the type of nonspecific lysis known to occur in the presence of lymphocyte agglutinins such as concanavalin A and phytohemagglutinins (24, 25).

To test for nonspecific lysis, P815-SV were compared with untreated P815 cells as target cells for cytotoxic T cells. These T cells were generated against irrelevant antigens by culturing DBA/2 spleen cells together with mitomycin C-treated C57BL/6 spleen cells. Such T cells should be specific for the $H\text{-}2^b$ antigens of the C57BL/6 stimulator cells and, as expected, they lysed untreated P815 cells to only a small extent (Fig. 4). P815-SV were not lysed to any greater extent (Fig. 4), however, indicating that treating the target cells with inactivated Sendai virus did not render them susceptible to nonspecific lysis.

In contrast, the untreated P815 cells were lysed in the presence of phytohemagglutinin (Fig. 4), indicating that under appropriate conditions nonspecific lysis did occur. Moreover, the P815-SV were readily lysed by syngeneic cytotoxic T cells generated against Sendai virus-coated cells (Fig. 4), demonstrating that the P815 cells were effectively coated with Sendai virus in this particular experiment. An absence of nonspecific cytotoxicity of Sendai virus-coated target cells was also observed in separate experiments with Sendai virus-

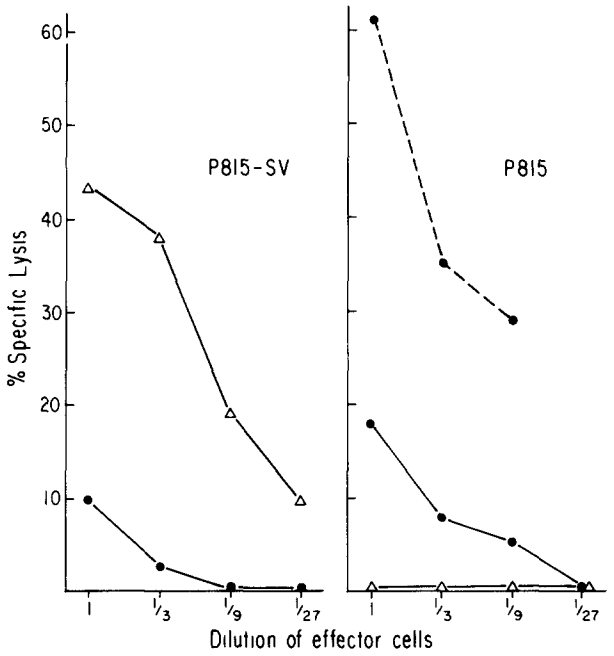

FIG. 4. Absence of lysis of target cells coated with inactivated Sendai virus in the presence of immunologically irrelevant cytotoxic lymphocytes P815-SV (left graph) or normal P815 cells (right graph) were used as target cells in assays of two separate populations of cytotoxic lymphocytes. Shown is the specific lysis by DBA/2 anti-C57BL/10 cytotoxic lymphocytes (—●—) generated by culturing DBA/2 spleen cells with mitomycin C-treated C57BL/10 spleen cells, and by Sendai virus-specific cytotoxic lymphocytes (—△—) generated by culturing spleen cells from immune DBA/2 mice with Sendai virus-coated syngeneic cells. Also shown (right graph) is the specific lysis of P815 cells by DBA anti-C57BL/10 cytotoxic lymphocytes in the presence of PHA, 10 μg/ml, (--●--).

coated EL4 (*H-2*^{b}) cells and C57BL/6 (*H-2*^{b}) cytotoxic cells generated against allogeneic H-2 antigens.

Involvement of the Major Histocompatibility Complex in the Lysis of Sendai Virus-Coated Target Cells. To investigate whether the major histocompatibility complex was involved in the lysis, spleen cells from B10.D2 (*H-2*^{d}) mice previously immunized with inactivated Sendai virus were cultured with Sendai virus-coated isologous cells and then assayed for cytotoxic activity against either P815 (*H-2*^{d}) or EL4 (*H-2*^{b}) cells, each coated with inactivated Sendai virus. The B10.D2 cytotoxic cells lysed P815-SV, but did not lyse Sendai virus-coated EL4 cells (Fig. 5).

The B10.D2 strain differs from the strain of origin of P815 cells, (DBA/2), at a large number of genetic loci, but both of these strains share the *H-2*^{d} haplotype. On the other hand the B10.D2 strain and the C57BL/6 strain, in which the EL4 tumor originated, are genetically very similar except for their *H-2* haplotypes. The C57BL/6 strain has the *H-2*^{b} haplotype; the B10.D2 strain has the *H-2*^{d} haplotype on a C57BL/10 genetic background. With B10.D2 cytotoxic T cells, the lysis of P815-SV correlated with identity at the major histocompatibility complex despite multiple differences elsewhere in the genome. Furthermore, the lack of lysis of Sendai virus-coated EL4 cells by B10.D2 cytotoxic T cells corre-

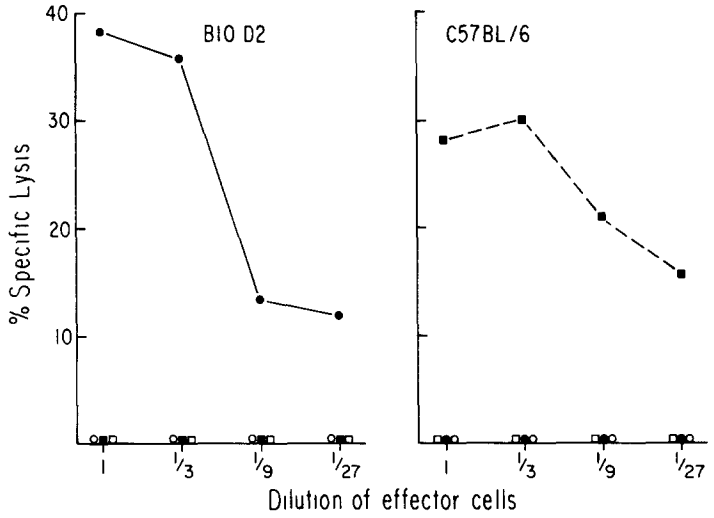

FIG. 5 Restriction by the major histocompatibility complex of the T-cell-mediated lysis of target cells coated with inactivated Sendai virus. Cytotoxic lymphocytes active against Sendai virus-coated target cells were generated as described above using spleen cells from immunized mice of the B10.D2 or C57BL/6 strains. The cytotoxic activity of these two sets of cultures was assayed against EL4 cells (□), EL4 cells coated with inactivated Sendai virus (■), P815 cells (○), and P815-SV (●). The left graph shows the specific lysis obtained with B10.D2 cultures (——); the right graph shows that obtained with C57BL/6 cultures (----)

lated with nonidentity at the major histocompatibility complex despite strong similarities elsewhere in the genome. These results suggest that, for lysis to occur, the cultured cells and the Sendai virus-coated target cells had to share *H-2* haplotypes.

This conclusion was further supported by the results of parallel assays of C57BL/6 cells which had been cultured with inactivated Sendai virus (Fig. 5). The C57BL/6 ($H\text{-}2^b$) cells lysed the syngeneic tumor EL4 ($H\text{-}2^b$) when it was coated with Sendai virus, but were ineffective against P815-SV ($H\text{-}2^d$). All of these data suggest that products of the major histocompatibility complex on Sendai virus-coated target cells are recognized by the specific cytotoxic T cells.

The Presence of Other Cytotoxic Activities in Cultures with Anti-Sendai Virus Activity. In experiments in which the cytotoxic activity against Sendai virus-coated tumor cells of the same *H-2* type was particularly strong and detectable with high dilutions of the cultured cells, we observed that there was also some cytotoxic activity against untreated tumor cells of a different *H-2* type. Fig. 6 shows the results of such an experiment. Spleen cells taken from immunized C57BL/6 mice ($H\text{-}2^b$) and cultured with Sendai virus-coated isologous cells were strongly lytic for Sendai virus-coated EL4 ($H\text{-}2^b$) cells but not untreated EL4 cells (Fig. 6). Cells from these cultures, however, also lysed both Sendai virus-coated and untreated P815 ($H\text{-}2^d$) cells. Similar results were obtained with spleen cells taken from immunized B10.D2 mice ($H\text{-}2^d$) and cultured with Sendai virus-coated isologous cells. These cultures, which were strongly lytic for P815-SV, but not for untreated P815 cells, also had detectable cytotoxic activity against both Sendai virus-coated and untreated EL4 cells (Fig. 6).

In this experiment, both the C57BL/6 and the B10.D2 cultured cells had some

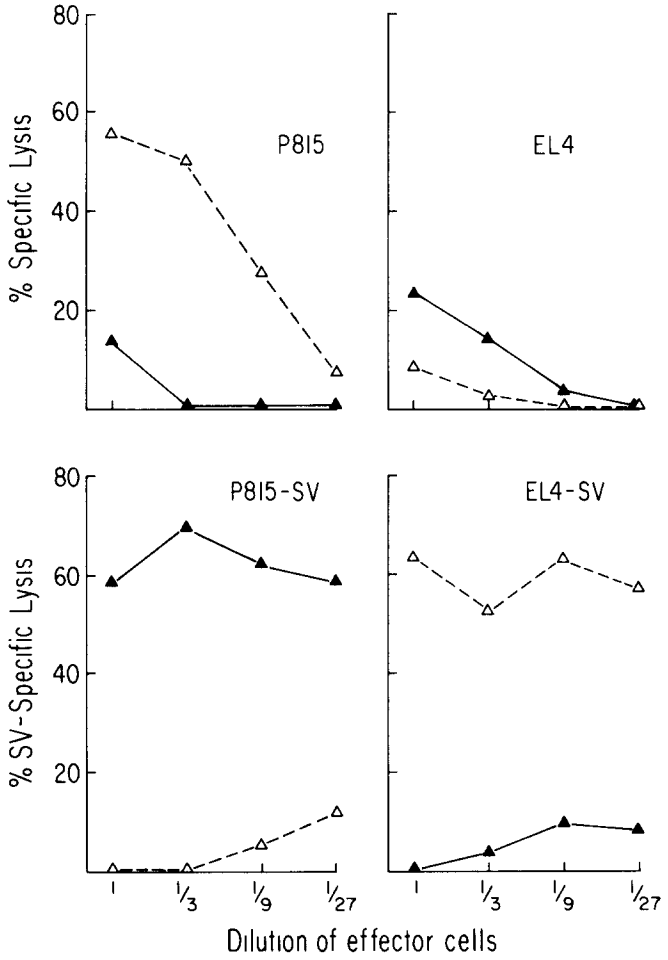

FIG 6 Lysis of allogeneic H-2 incompatible tumor cells by cultures having high cytotoxic activity against H-2 compatible cells coated with inactivated Sendai virus (SV). Spleen cells from immunized B10.D2 (—▲—) or C57BL/6 (--△--) mice were cultured with mixtures of inactivated Sendai virus and syngeneic cells as before Cultures were assayed for cytotoxic activity against P815 and EL4 cells, and against P815 and EL4 cells coated with inactivated Sendai virus. Specific lysis of P815 and EL4 cells not coated with virus is shown in the top graphs. The lower two graphs show the high titer of Sendai virus-specific lysis assayed on P815-SV and EL4 cells coated with inactivated Sendai virus (EL4-SV) and calculated as described in the text

cytotoxic activity against Sendai virus-coated target cells of a different *H-2* type (P815 or EL4 cells, respectively). To estimate the proportion of this lytic activity which was specific for cells coated with virus, it was necessary to correct for the lysis of P815 or EL4 cells which had not been coated with virus. The specific lysis of uncoated target cells was therefore subtracted from the specific lysis of Sendai virus-coated targets. Fig. 6 shows that, when the Sendai virus-specific lysis is calculated in this manner, these results clearly confirm the previous conclusions: Sendai virus-specific lysis of P815-SV ($H\text{-}2^d$) was much greater with the B10.D2 ($H\text{-}2^d$) spleen cells than with the C57BL/6 ($H\text{-}2^b$) spleen cells, and

Sendai virus-specific lysis of Sendai virus-coated EL4 ($H\text{-}2^b$) cells was much greater with C57BL/6 ($H\text{-}2^b$) spleen cells than with B10.D2 ($H\text{-}2^d$) spleen cells.

The lysis of uncoated tumor cells of allogeneic *H-2* types appeared to correlate with the presence of a vigorous response against Sendai virus. High levels of cytotoxicity against *H-2* compatible targets coated with Sendai virus were obtained in a tertiary response produced by culturing spleen cells from immunized C57BL/6 mice with inactivated Sendai virus. After 7 days an additional 10^6 isologous spleen cells and 0.03 ml of inactivated Sendai virus were added, and the cultures were continued for a further 5 days. The cultures had high levels of cytotoxicity against Sendai virus-coated EL4 cells, but not uncoated EL4 cells, and also lysed uncoated P815 cells.

The cytotoxic activity against nonvirus-coated allogeneic target cells probably reflects the presence of a polyclonal response (26), perhaps stimulated by nonspecific factors released during a vigorous specific response. While the generation of cytotoxic activity for uncoated allogeneic target cells made determination of the Sendai virus-specific response more complicated, the cytotoxic activity specific for target cells coated with inactivated Sendai virus was still restricted by the *H-2* type of the target cell.

The Kinetics of Lysis of Target Cells Coated with Inactivated Sendai Virus. We next examined the kinetics of lysis of Sendai virus-coated target cells in order to determine whether there was a long latent or synthetic period before Sendai virus-coated target cells became susceptible to lysis. Target cells were labeled with ^{51}Cr and then coated with inactivated Sendai virus by incubation at 4°C for 30 min. The virus-coated target cells were then added to centrifuge tubes together with a population of cultured spleen cells containing cytotoxic T cells specific for Sendai virus, and the mixture was gently centrifuged. All of these procedures were carried out at 4°C. The mixtures of cytotoxic T cells and Sendai virus-coated target cells were then incubated at 37°C for varying times to allow lysis to occur. Lysis was halted by raising the temperature to 43°C (19, 20). The release of ^{51}Cr from lysed cells was allowed to continue for 60 min at 43°C, after which the tubes were centrifuged and aliquots of the supernates taken for counting.

The results of a typical experiment are shown in Fig. 7. Significant lysis of Sendai virus-coated target cells was detectable even after incubation with the specific cytotoxic T cells for only 30 min. If the line connecting the 120, 60, and 30 min time points in Fig. 7 is extrapolated to the abscissa, it would intersect at the 15-min point, suggesting a lag in the onset of lysis. A similar lag was seen in other experiments in which the cytotoxic T cells were directed against H-2 antigens on allogeneic target cells and in which there was presumably no lag in the appearance of antigen on the targets. Thus, the small delay in the onset of lysis is probably related to the state of cytotoxic T cells early in the assay, rather than the delayed appearance of antigens on the target cells. In any case, these experiments establish that Sendai virus-coated cells were suitable targets for specific cytotoxic T cells after only 30 min at 37°C.

Lack of Synthesis of New Proteins in Cells after Coating with UV-Inactivated Sendai Virus. Although these experiments tended to rule out protein synthesis as a major factor, it was important to determine directly whether any virus-directed protein synthesis occurred in the target cells after coating with UV-

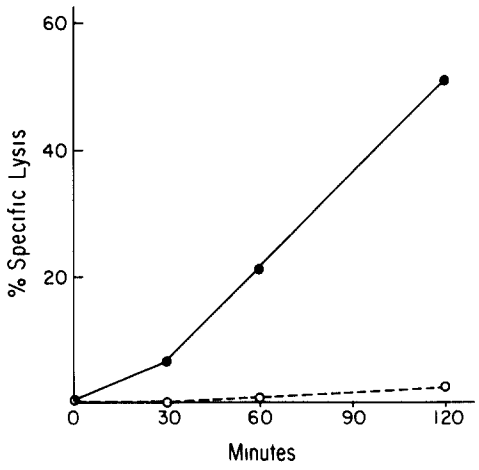

FIG 7 The kinetics of lysis of target cells coated with inactivated Sendai virus The specific lysis by cytotoxic lymphocytes directed against Sendai virus determinants was measured at various time points using as targets normal P815 cells (--○--), or P815-SV (—●—). The cytotoxic lymphocytes specific for Sendai virus were generated by culturing spleen cells from immune DBA/2 mice with inactivated Sendai virus and syngeneic cells.

inactivated Sendai virus. P815 cells were coated with UV-inactivated Sendai virus, under the same conditions used for preparing target cells for the cytotoxic assay. The virus-treated cells were cultured for 16 h, pulsed for a further 2 h with [^{3}H]leucine, and then washed and disrupted with SDS (5%). Similar samples were prepared from untreated P815 cells that were cultured in parallel and pulsed with either [^{35}S]methionine or [^{3}H]leucine. Portions of these [^{3}H]leucine- and [^{35}S]methionine-labeled extracts were pooled, and the mixtures were subjected to electrophoresis on 10% polyacrylamide disk gels. Fig. 8 shows the distribution of ^{3}H and ^{35}S in the fractions of a gel loaded with a mixture of an [^{3}H]leucine-labeled extract of P815 cells which had been coated with UV-inactivated Sendai virus and a [^{35}S]methionine-labeled extract of normal P815 cells. There was no significant increase in the ratio of ^{3}H to ^{35}S in any gel fraction. Comparison of the gels in which equal amounts of the [^{3}H]leucine-labeled extracts of normal P815 cells and virus-treated P815 cells were run in parallel, also failed to reveal any differences between the normal and virus-treated cells.

To investigate whether synthesis of a new protein might only be detectable early after contact of the cells with UV-inactivated Sendai virus, P815 cells were coated with inactivated virus and pulsed with [^{3}H]leucine without any preliminary period of culture. Control cells were pulsed with [^{35}S]methionine, and samples of the Sendai virus-coated and normal cells were processed and electrophoresed on polyacrylamide gels as before. Again, there was no evidence for an increased ratio of ^{3}H to ^{35}S in any fraction of the gel.

In further experiments, normal P815 cells or P815 cells previously coated with either UV-inactivated or infectious Sendai virus were cultured for 10 h and labeled with [^{35}S]methionine for 2 h. Extracts of these cells were run in parallel on high-resolution slab gels which were stained with Coomassie Blue and then processed for autoradiography (Fig. 9). In contrast to the results obtained with

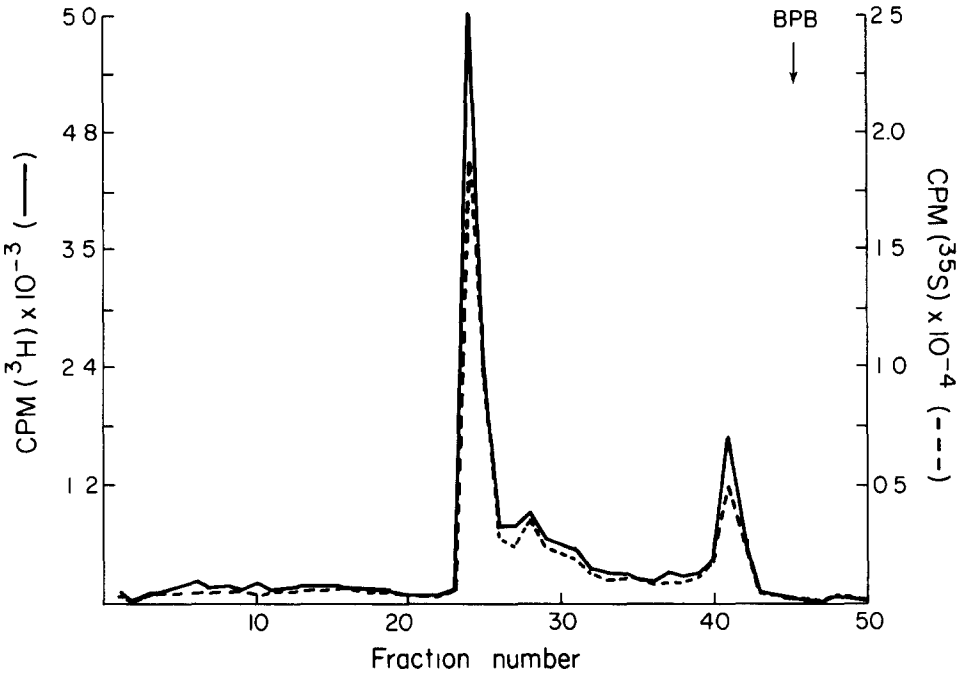

FIG 8 Protein synthesis in P815 cells coated with UV-inactivated Sendai virus. An extract of P815 cells that had been coated with inactivated Sendai virus, cultured for 16 h, and labeled by incorporation of [^{3}H]leucine, was mixed with an extract of normal P815 cells that had been cultured in parallel and labeled by incorporation of [^{35}S]methionine. The mixture was loaded on a 10% polyacrylamide gel and, after electrophoresis, the gel was sliced and counts per slice determined in a liquid scintillation counter Solid lines show the counts due to disintegration of the ^{3}H incorporated into the cells coated with inactivated Sendai virus; broken lines show counts due to disintegration of the ^{35}S incorporated in control P815 cells BPB, Bromophenol blue dye.

infectious virus, there was no evidence for the synthesis of new protein in the cells coated with UV-inactivated virus, confirming the results obtained using the disk gels. Similar experiments using cells incubated for either 1.5 or 16 h after coating with inactivated virus and labeled with [^{3}H]leucine also failed to show evidence for the synthesis of new protein.

These results suggest that the UV-inactivated Sendai virus was neither forming new virion proteins, nor directing the synthesis of other new proteins in virus-coated target cells.

Discussion

We have demonstrated that virus-specific cytotoxic T cells can be generated in vitro by culturing spleen cells with inactivated Sendai virus. Such cytotoxic T cells lysed target cells which had been coated with the inactivated virus. Sendai virus-specific lysis was restricted by the *H-2* haplotype of the target cell, and the target cells were susceptible to specific lysis as early as 30 min after coating with the inactivated virus. Moreover, there was no evidence of the synthesis of new viral proteins after treatment with the inactivated virus.

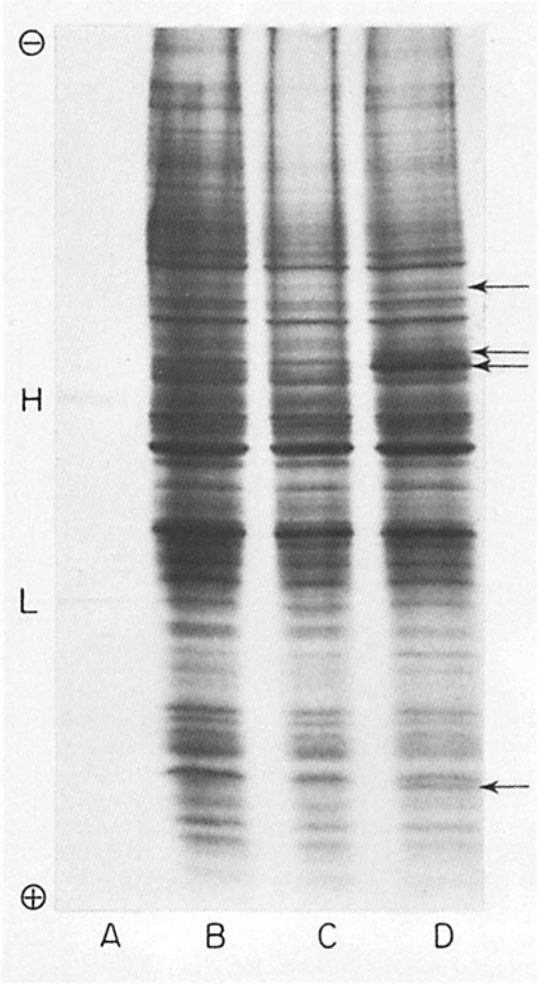

FIG. 9. High resolution comparison of proteins from normal and virus-treated cells on slab gels Normal P815 cells or P815 cells coated with inactivated or infectious Sendai virus were cultured for 10 h and pulsed with [^{35}S]methionine for a further 2 h. Autoradiographs are of (A) markers, the heavy chain (H), mol wt 50,000 and the light chain (L), mol wt 23,000, of the IgG myeloma protein MOPC 21; (B) P815-SV, (C) untreated P815 cells; (D) P815 cells coated with infectious Sendai virus Arrows indicate bands that appear in cells treated with infectious virus but not in untreated cells or in cells treated with UV-inactivated virus.

This in vitro system for the generation of virus-specific cytotoxic T cells offers new opportunities for an analysis of cell-cell interactions in the initiation of immune responses against viruses. Furthermore, the demonstration that immunological memory for a cytotoxic T-cell response can be generated by in vivo immunization with an inactivated virus (Fig. 1), is in accord with the observation of Shellam et al. on oncornaviruses (27), and may be relevant to the design of vaccination procedures against these kinds of viruses. In this discussion, however, we shall concentrate on the effector phase of the response and particularly on the implications of the lysis of target cells coated with inactivated Sendai virus.

The observation that the H-2 antigens of virally infected cells are recognized by virus-specific cytotoxic T cells (1–5) raises the question of the nature of the structures recognized by cytotoxic T cells. The one-receptor hypothesis proposes that the virus-specific cytotoxic T cell recognizes a complex antigenic determinant specified both by the virus and by the H-2 genes of the host cells (1, 7). Because the Sendai virus used to coat the target cells was inactivated by UV-irradiation, the present experimental system excludes several possible mechanisms for conversion of cells into suitable targets for virus-specific cytotoxic T cells. It appears that replication and budding of the virus are unnecessary for the production of an appropriate target antigen. The cytotoxic assay was of relatively short duration, and lysis was detected as early as 30 min after the target cells had been coated with inactivated virus. This suggests that the virus-specific determinant recognized by the T cell was either synthesized very early after contact of inactivated virus with the cell or that this determinant was actually a preformed constituent of the virion that had adsorbed onto, or had been incorporated into, the target cell membrane.

The possibility that the T cell recognizes a virus- or H-2-specific antigen that is synthesized less than 30 min after contact of the virus with the cell seems unlikely. Sendai virus is a negative-stranded RNA virus, and the initial event after entering the cells is transcription of this virion RNA to messenger RNA (28). In infection by active Sendai virus, new viral protein is detectable only after a latent period of several hours (29). Furthermore, we could find no evidence for the synthesis of new proteins in tumor cells coated with UV-inactivated Sendai virus (Figs. 8 and 9). With the qualification that these techniques might not detect new proteins formed in very small amounts, it seems highly unlikely that the target for the cytotoxic T cells is a newly synthesized protein.

An alternative possibility is that the virion contains an enzyme capable of modifying host structures in a specific way. For example, Sendai virus might contain a glycosyltransferase. There is no evidence to support this view; in the case of paramyxoviruses such as Sendai virus, the glycolipids (30) and probably the glycoproteins (31) are glycosylated by host enzymes.

For these reasons, the observation that target cells very rapidly become susceptible to lysis by virus-specific T cells after coating with inactivated Sendai virus (Fig. 8) seems more compatible with the notion that the virus-specific determinants are on preformed virion antigens which have been incorporated into the target cell membrane by adsorption or fusion of the virus. This idea is especially attractive because Sendai virus antigens are known to persist in association with cell membranes after exposure of the cells to the virus (32, 33). Work is currently in progress to test this hypothesis even more stringently, using isolated viral proteins.

The conclusion that the virus-specific component recognized by specific cytotoxic T cells can be contributed by a virion antigen is consistent with observations that viral particles or purified viral antigens can inhibit T-cell-mediated cytolysis of cells infected with oncornaviruses (27). On the basis of these experiments, Shellam and colleagues (27) have suggested that the inhibition of cytotoxicity is due to the binding of virion antigen alone by the cytotoxic T cell. The inhibition could, however, also be due to competition by lymphocytes of the

effector population that have adsorbed virus and therefore become virus-coated targets similar to those we have studied with Sendai virus.

It is pertinent to compare our data on the nature of the viral antigens recognized by cytotoxic T cells with the reports of two other groups (34, 35). Both groups worked with pox viruses and both reached the conclusion that viral replication was not essential for the production of suitable target antigens. The relevant antigens appeared to be proteins synthesized early after viral infection, and lysis of infected cells by virus-specific T cells was not demonstrable until 3 h after infection (34). Furthermore, inhibitors of protein synthesis blocked the appearance of the virus-specified antigens which were synthesized during the first 1–2 h after infection. Thus, in contrast to our results, the studies of Ada and colleagues suggested that simple coating of target cells with virus particles did not convert them to suitable targets for virus-specific T cells.

Several factors could account for the differences between the results with pox viruses and our own observations with Sendai virus. First, some caution must be exercised in comparing the two assay systems used. In the present system, both the stimulator cells and the target cells may be simply virus-coated cells. In the pox virus systems, the cytotoxic T cells were generated by an in vivo infection, in which virus-coated cells may not necessarily have been the most prevalent or potent immunogens. It would therefore be of interest to see whether cytotoxic T cells generated in a natural in vivo infection by Sendai virus are active against Sendai virus-coated target cells. Second, the Sendai virus may differ from pox viruses because of its particular ability to fuse and integrate with cell membranes (32, 33, 36). Third, the exact manner of the association of viral proteins with the cell membrane may be critical for the formation of an antigenic determinant which is immunogeneic for cytotoxic T cells. Thus, we have suggested that the cytotoxic T cell recognizes a hybrid determinant formed by the physical association of a viral antigen and an H-2 antigen (7, 17). In the case of pox viruses, it may be that only an early protein and not virion antigens are able to associate with H-2 antigens.

The present experiments do not in themselves distinguish between the one-receptor and two-receptor hypotheses for the recognition of virally infected cells by cytotoxic T cells. They do suggest, however, that if the one-receptor hypothesis is valid, the determinant that is seen by the T-cell receptor is formed by the physical association of preformed H-2 and viral antigens. Moreover, the implication that readily purified virion antigens can form part of the target antigens for cytotoxic T cells offers new opportunities for investigating both the association of viral antigens with H-2 antigens and the binding of these antigens to the T-cell receptor.

Summary

Cytotoxic T cells specific for Sendai virus were generated by culturing murine spleen cells in vitro together with UV-inactivated Sendai virus. In vivo immunization of donor mice with UV-inactivated Sendai virus resulted in an in vitro secondary response of increased magnitude. Cytotoxic activity was demonstrated in a short-term ^{51}Cr-release assay, using syngeneic tumor cells which had been coated with inactivated Sendai virus by incubation at 4°C for 30 min.

The lysis of Sendai virus-coated target cells was restricted by the *H-2* haplotype of the target cells, suggesting that the H-2 genes of the target cell contributed to the specificity of the lysis. Kinetic experiments showed that susceptibility to lysis by cytotoxic T cells specific for Sendai virus appeared within 30 min after coating target cells with inactivated virus. Furthermore, there was no detectable synthesis of new proteins in cells treated with UV-inactivated Sendai virus.

For these reasons, we suggest that neither viral replication nor the synthesis of new proteins are necessary for the production of the antigen recognized by cytotoxic cells specific for Sendai virus. We infer that the virus-specific component on the target cells is probably a preformed virion antigen adsorbed onto or integrated into the cell membrane. These results imply that, if the cytotoxic T cell recognizes a single antigenic determinant specified both by viral and H-2 genes, this determinant is formed by the physical association of H-2 and Sendai virus antigens rather than by their alteration during the processes of synthesis.

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