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Generation of virus-specific cytotoxic T cells *in vitro* II. Induction requirements with functionally inactivated virus preparations*

Using noninfectious Sendai virus preparations after selective enzymatic digestion of either of the two viral envelope glycoproteins, it was possible to study the effect of different virion-cell membrane interactions on virus-specific cytotoxic T lymphocyte (CTL) induction *in vitro*. Three different virus preparations having capacity for virus-cell fusion, for virus-cell adsorption or lacking the ability to bind to cell membranes, were all active in the generation of virus-specific primary and secondary cytotoxic T cells, when added to the culture. Investigations on the responder cell requirements during CTL induction revealed that activation by addition of virions lacking the capacity to bind to cells was sensitive to the depletion of adherent cells. When virions with fusion and binding capacity were presented on tumor stimulator cells, different requirements with respect to adherent cells were obtained in the primary and secondary CTL response to Sendai virus. The data indicate that different viral antigen-cell membrane interactions govern the activation phase and effector phase of antigen-primed T cell populations, while sensitization of unprimed cells is dependent on the presence of adherent, perhaps antigen-presenting cells.

1 Introduction

Virus-specific cytotoxic T cells (CTL) are restricted by the major histocompatibility complex (MHC) and specific for the sensitizing virus. In order to effect lysis of target cells, both antigenic determinants recognized by the CTL, *i.e.* the H-2 antigens and the X-antigens of the virus, must be presented as integral components of the membrane of the same target cell [1, 2]. Infectious virus, injected *in vivo* in order to trigger CTL, integrates surface antigens into the cell membranes and thus provides a situation identical to that required for the CTL effector activity. However, several investigators have recently shown that noninfectious viral preparations will induce virus-specific CTL *in vivo* [3–5] and *in vitro* [1, 2, 6–12]. If the maturation of virus-specific precursors into CTL is initiated only after presentation of viral antigens together with cell membrane structures, then the various virus cell membrane interactions should play a crucial role in T cell activation.

This report presents data using preparations of noninfectious, β-propiolactone-inactivated Sendai virus (β-PL-SV) which is active in virus-cell adsorption and virus-cell fusion. After selective proteolytic digestion, we obtained virion preparations with defective capacities for interaction with cell membranes. Virions treated with trypsin retain the capacity to adsorb to cells but do not fuse with the cell membrane, while those treated with the V8 protease from *Staphylococcus aureus* lack both the adsorption and the fusion capacity. These virion preparations enabled us to study the requirements for virion-cell membrane interactions during the induction of primary and secondary CTL *in vitro*. The data indicate that acti-

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* Supported by the Deutsche Forschungsgemeinschaft Ko 571/5 and Ko 571/7.

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Abbreviations: SV: Sendai virus **β-PL-SV:** SV inactivated with β-propiolactone **CTL:** Cytotoxic T lymphocytes **ELISA:** Enzyme-linked immunosorbent assay **F:** Envelope glycoprotein of SV associated with fusion activity **HANA:** Envelope glycoprotein of SV with hemagglutination and neuraminidase activity **HAU:** Hemagglutination unit **Try-SV:** Inactivated SV after digestion with trypsin **V8-SA-SV:** Inactivated SV after digestion with V8 protease of *Staphylococcus aureus*

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vation phase and effector phase of secondary H-2-restricted virus-specific T cells have different requirements for presentation of viral antigens on cell membranes. Unprimed spleen cell populations have additional requirements for adherent cells which seem to be active in antigen processing.

2 Materials and methods

2.1 Animals

DBA/2 and BALB/c strain mice (H-2^d) were purchased from Gl. Bomholtgård, Ry, Denmark, or were raised in our own breeding facility (DBA/2 HD). Mice were used at 6–10 weeks of age.

2.2 Viruses

Sendai virus (SV) and Influenza A/Victoria virus (H_3N_2) were propagated, titrated, purified and inactivated as described [1, 13, 14]. β-PL-SV (2 mg in 1 ml of the appropriate buffer) was digested with proteolytic enzymes as described previously [1]. Trypsin treatment [15]: (8 µl, 5 mg/ml^{-1}; TPCK-treated, Worthington Biochemical Corp., Freehold, NJ) for 1 h at 37 °C in 0.5% ammonium hydrogencarbonate buffer, pH 8. Treatment with V8 protease from *Staphylococcus aureus:* (50 µl, 1 mg/ml^{-1}; Miles Labs., Kankakee, IL) for 18 h at 37 °C in 50 mM ammonium hydrogencarbonate buffer, pH 8.

The capacity of the virus preparations to induce target cell formation was measured in the cytolysis assay [13]. Hemagglutinin, neuraminidase, hemolytic activities and cell fusion were tested as described previously. As reported [1], virus preparations treated with trypsin (Try-SV) had hemagglutination and neuraminidase activities comparable to β-PL-SV, but lacked the fusion and the hemolytic activity (F^-). The preparation digested with V8 protease from *S. aureus* (V8-SA-SV) still contained the F glycoprotein, but the hemagglutinin-neuraminidase glycoprotein spike was removed from the virion ($HANA^-$).

2.3 Immunizations

Mice were injected intraperitoneally (i.p.) with 100 hemagglutinin units (HAU) of infectious virus. Three to 10 weeks afterwards, spleens were removed and lymphocytes were prepared for tissue culture. Antiserum to SV was raised in DBA/2 mice by three weekly i.p. injections of 100 HAU SV. Serum was obtained 5 days after the final injection and tested by hemagglutinin inhibition (titer: 2.5×10^{-3}) and enzyme-linked immunosorbent assay (ELISA) [16] (titer: 3.5×10^{-5}).

2.4 Media

Cells were cultured in RPMI 1640 medium with L-glutamine (2 mM final concentration), streptomycin and penicillin (50 units/ml), 2-mercaptoethanol (2×10^{-5} M) and 10% fetal calf serum.

2.5 Cell cultures

P815-X2 (H-2^d) and Eb (H-2^d) tumor cells, which carry non-cross-reactive tumor-specific antigens [17], were grown in medium at a concentration of 2×10^5 cells/ml with medium change after every 48 h. Mouse spleen cells or lymph node cells were suspended in medium at a concentration of 4×10^6 cells/ml. Cells were cultured in multi-dish culture trays (FB-24 Tc, Linbro Chemicals, New Haven, CT) or in plastic tissue culture flasks of different sizes. If desired, cells were depleted of erythrocytes by lysis in 0.184 M NH_4Cl or by separation on a Ficoll-Hypaque gradient. B cell depletion was achieved through nylon wool column passage [18]. Macrophages and adherent cells were removed by passage of cells through Sephadex G-10 columns. Phagocytic cells in the cultures were destroyed by addition of 100 µg silica/ml (kindly provided by Dr. Lemke, Institut für Genetik, Köln, FRG) [19].

2.6 ^{51}Cr-release assay

The conditions of the ^{51}Cr-release assay and the calculation of data have been described previously [10]. All values are the mean percent specific ^{51}Cr release of triplicate wells. The standard errors of the means were always less than ± 5% and are omitted from the figures for clarity.

3 Results

3.1 Induction of a SV-specific secondary CTL response with different virus preparations

We were interested in studying the correlation of induction of virus-specific CTL with the capacity of virions to interact with cells. We have prepared modified Sendai virions, β-PL-SV, Try-SV (F^-) and V8-SA-SV ($HANA^-$) which differ in the functional activities of the surface glycoproteins. β-PL-SV is active in virus-cell binding and fusion, the F^- preparation only attaches and binds while the $HANA^-$ preparation does not adsorb to cells, due to lack of the HANA glycoprotein.

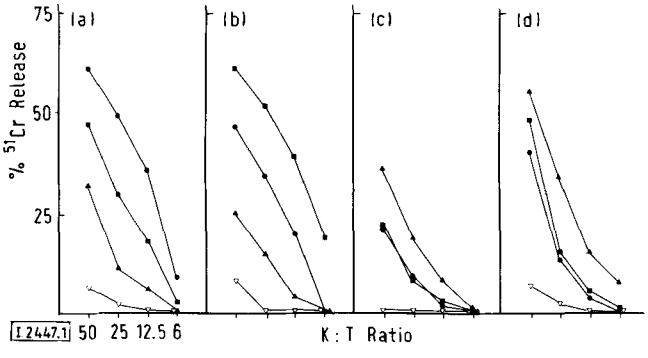

Figure 1. Restimulation of sensitized spleen cells with different virus preparations. Responder cells used were P815 cells infected with 100 HAU SV or 100 HAU influenza A/Victoria/10^6 cells. Target cells were incubated with virus for a period of 4 h before test. Cytolytic activity of responder cells on SV-infected P815 target cells was tested after 5 days of incubation with: (●–●) 0.2 HAU/ml infectious SV (a) or 0.1 µg/ml virus protein of the inactivated preparations (β-PL-SV (b), V8-SA-SV (c) and Try-SV (d)). (■–■) 2 HAU/ml SV or 1 µg/ml virus protein; (▲–▲) 20 HAU/ml SV or 10 µg/ml virus protein; (▽–▽) cytolytic activity of the responder cells incubated with 20 HAU SV/ml or 10 µg/ml virus protein, respectively, on P815 target cells infected with influenza virus. Killer cells and target cells were coincubated for 4 h.

Spleen cells from DBA/2 strain mice, primed four weeks previously with 100 HAU of SV, were restimulated *in vitro* for 6 days by addition of the various virus preparations to the culture and then tested for cytotoxic activity. The results in Fig. 1 show that, as has been reported previously [10], both infectious and β-PL-SV were active in generating secondary cytotoxic effector cells. In both cases, the virions are capable of fusing with the plasma membrane, and the viral glycoproteins are presented as integral membrane components of cells in the culture.

However, in contrast to the results obtained previously for formation of target cells [1], virions which had been digested with trypsin to selectively inactivate the fusion protein (F^-) or with *S. aureus* V8 protease to remove the hemagglutinin-neuraminidase protein ($HANA^-$) were also active in stimulating a secondary CTL response.

Quantitation of the amounts of viral protein required in order to generate comparable cytotoxic activity (by calculation of lytic units) revealed that trypsin treatment decreased the stimulating capacity 2–3-fold and V8 protease treatment about 20-fold. In contrast, the fusion activity of Try-SV and the hemagglutinin-neuraminidase activities of V8-SA-SV were decreased at least 100-fold [1]. When these preparations (Try-SV, V8-SA-SV) were checked for target cell formation, which can be taken as a sensitive indicator for virus-cell fusion [1, 13], up to 500-fold excess of the amount of β-PL-SV required for target cell formation was tested and found to be negative. Therefore, if there was any fusion capacity left in these preparations, it had to be more than 500 times less than in β-PL-SV (data not shown).

CTL, generated with all of these viral preparations, were specific for SV and did not lyse syngeneic target cells infected with influenza A strain viruses (Fig. 1). Furthermore, CTL, generated with these preparations, were restricted by the MHC and were sensitive to treatment with antiserum to Thy-1.2 and complement (data not shown).

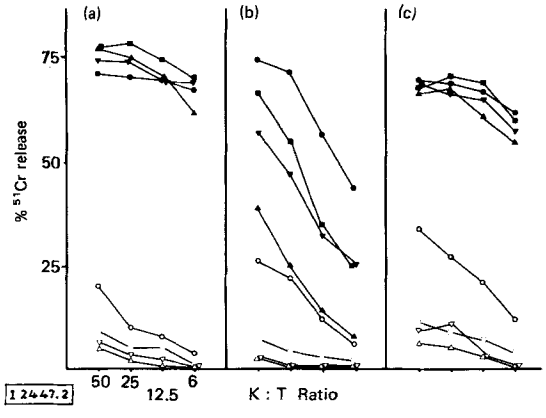

Figure 2. Time course of primary induction of virus-specific CTL with different virus preparations. Responder cells (DBA/2/HD normal) were incubated with the viral antigens (1 μg/ml β-PL-SV (a), 10 μg/ml V8-SA-SV (b), 10 μg/ml Try-SV (c)) for different periods of time, followed by washing, resuspension in medium containing 10% antiserum to SV for 30 min, followed again by threefold washing and resuspension in antigen-free medium. Incubation time with antigen: ▲, △ 1 day, ▼, ▽ 2 days, ■, □ 3 days, ●, ○ 5 days. Effector cells generated after 5 days were tested on SV-infected P815 (open symbols) and uninfected P815 control (closed symbols) cells.

3.2 Induction requirements of virus-specific primary responses with SV preparations

In a preceding paper [10], we have described the conditions of the *in vitro* induction of a primary CTL response to SV. Lymphocytes from mice negative for antibody to SV were suspended in tissue culture medium supplemented with 0.1 μg/ml β-PL-SV, or 1 μg/ml V8-SA-SV or 1 μg/ml Try-SV, respectively. Cells were incubated either over the whole period of 5 days in medium containing the antigen, or the cells were resuspended after different intervals into antigen-free medium after 3-fold washing and additional treatment of cells with anti-SV virus antibody for 30 min at 37 °C. Using optimal antigen concentrations, all three virus preparations, β-PL-SV, Try-SV and V8-SA-SV were active in the generation of a primary CTL response after 5 days (Fig. 2). Thus, SV preparations, lacking fusion activity or even inactive in cell adsorption, are capable of eliciting a primary CTL response to SV. In this experiment, the unprimed cell populations of BALB/c mice generated CTL with more efficient cytotoxic activity than the SV-primed populations from DBA/2 mice in the previous experiment (Fig. 1). This is not interpreted to mean that primary responses always yield a higher cytotoxic activity. Comparing several experiments of primed and unprimed cell populations, the average of cytolytic activity generated in primed cell populations is superior to that of unprimed spleen cells.

Although all three virion preparations induced a T cell response when incubated together with responder cells for 5 days, the time course of induction varied. With virions active in fusion and/or cell adsorption, a cytotoxic response was generated after incubation of responder cells with virus for a period as short as 1 h. Removal of antigen by washing or incubation with antibody after the initial binding did not interfere with CTL induction. Incubation with specific antibody blocks CTL generation only if the antibody is added before virus-cell binding has taken place [10]. Induction of primary CTL with V8-SA-SV, which lacks the HANA glycoprotein and cannot bind to cells, seems to occur by a different mechanism. Removal of unbound antigen from responder cells after various intervals of time showed that the duration of antigen presence in medium is crucial. The generated cytolytic activity increased with the time the antigen was present in the culture and reached an equivalent level to those cultures incubated with the other preparations only after 5 days. For this, there are at least two explanations: (a) the viral antigens in suspension are less active inducers of a T cell-mediated response, and washing reduces the antigen concentration below a required threshold; (b) the unbound antigen is not active in CTL triggering and requires the presentation on cells within the culture.

3.3 Role of adherent and phagocytic cells in SV-specific CTL induction

In order to investigate the role of putative antigen-presenting cells, primary and secondary induction of SV-specific CTL was carried out using purified cell populations. T cells were enriched by passage of spleen cells through a nylon wool column. Depletion of adherent cells from this T cell-enriched fraction was achieved by passage of cells through a Sephadex G-10 column. Alternatively, the nylon wool column-enriched T cells were cultured *in vitro* with viral antigens in the presence of silica which is selectively toxic for phagocytic cells without

reducing the viability of lymphocytes *in vitro* [19]. The final experimental protocol combined these techniques, using nylon wool-passaged T cells depleted of adherent cells by Sephadex G-10 column and incubated with viral antigens in the presence of silica. The results are shown in Fig. 3 a and b. Spleen cells from primed BALB/c mice depleted of B cells responded well to β-PL-SV, V8-SA-SV and Try-SV. Similarly, in previous experiments carried out to define the Lyt subsets which participate in the generation of SV-specific CTL [10], no qualitative differences in the removal of nylon wool-adherent cells on CTL generation by the virus preparations was observed. Further separation of macrophages by Sephadex G-10 column passage, or inactivation of phagocytic cells by the presence of silica, or both methods combined, did not affect the response to β-PL-SV and Try-SV. Decreased cytolytic activity was observed in the macrophage-depleted or macrophage-inactivated cultures using V8-SA-SV preparations as antigen.

When selected lymphocyte populations from unprimed BALB/c mice were tested, significant differences were observed. As seen with primed populations, cytotoxic responses to β-PL-SV and Try-SV were generated in populations enriched for T cells and depleted of phagocytic cells. In contrast, T cell populations depleted of phagocytic cells were not sensitized by V8-SA-SV. These results support the second explanation proposed for the results found with this antigen preparation in the time course experiment: viral antigens in suspension, which are unable to adsorb and/or fuse to cells, are also unable to activate antigen-specific T cells unless adherent and/or phagocytic cells are present in the culture. The suggestion that macrophages are actively involved in the presentation of viral antigens is also consistent with our previous data [20].

3.4 CTL response to virions on tumor stimulator cells

We have previously described [1] that Try-SV, after binding to P815 tumor cells, does not render these cells susceptible to SV-specific T cell-mediated lysis. When added to the culture medium, both Try-SV and V8-SA-SV were active in CTL generation. This suggested that integration of viral proteins into membranes was not a prerequisite for T cell activation. Because we could not exclude that these antigens are processed differently on spleen cells, we tested P815 tumor cells modified by the SV preparations as stimulators. This protocol is not readily applicable to V8-SA-SV since the majority of these virions without binding capacity were removed from the cells by the washing procedure after virus tumor cell incubation. The interpretation of results, using P815 cells incubated with Try-SV, is limited by the possibility that due to the neuraminidase activity, the virions detach from the P815 cells and are presented on other cells. Since similar constraints apply to the P-815-β-PL-SV stimulator cells, it cannot be

Table 1. Tumor stimulator cells and virus-specific CTL activation

Stimulator cells[a]	Activity of responder cells[b] on Eb-SV[c] target cells			
	SV-sensitized		Unprimed	
	T cell-enriched	adherent cell-depleted	T cell-enriched	adherent cell-depleted
P815	3.9[d]	4.1	3.4	5.5
P815 β-PL-SV	31.3	19.4	29.8	4.8
P815 Try-SV	22.9	15.4	–0.5	5.0
P815-V8-SA-SV	3.8	5.0	4.2	4.4

a) Stimulator cells were prepared by 1 h incubation of 1×10^6 3000 rd irradiated P815 cells with 1 μg β-PL-SV, 10 μg Try-SV or 10 μg V8-SA-SV, followed by washing of cells.
b) Stimulator : responder ratio was 1 : 2. Effector cells were tested on day 6.
c) Effector : target cell ratio was 20 : 1. Spontaneous release from Eb-SV target cells was 12%.
d) Results are given in % specific release after subtraction of activity on non-virus-modified Eb cells.

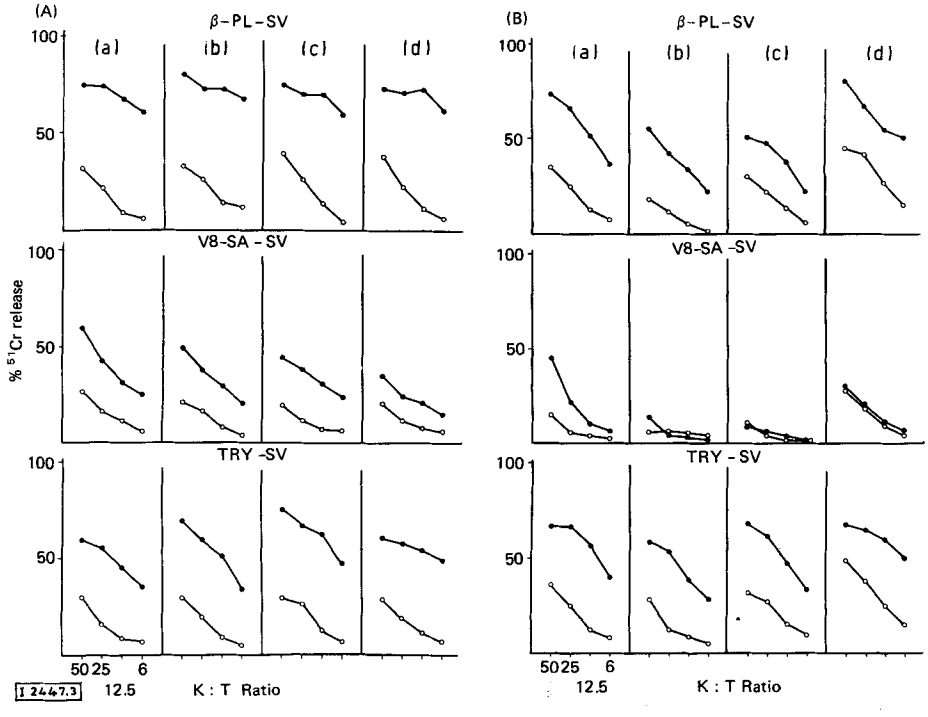

Figure 3. Effect of depletion of adherent cells and phagocytic cells on the induction of primary and secondary SV-specific CTL *in vitro,* using different virus preparations. Cell separation: (a) nylon wool column (NWC); (b) NWC + Sephadex G-10 column; (c) NWC + silica; (d) NWC + silica + Sephadex G-10 column. Responder cells: (A) BALB/c primed with 100 HAU infectious SV; (B) normal BALB/c cells. Target cells: P815 (○); P815-SV (●). S: Stimulating antigens were used in the concentrations: 1 μg/ml β-PL-SV, 10 μg/ml V8-SA-SV, 10 μg/ml Try-SV.

excluded that these cells may fuse with cells in culture so that the antigen may be re-expressed on other cells.

Despite these limitations, the findings obtained with these stimulator cells are clear (Table 1). Virions with fusion and with binding capacity are capable of activating antigen-primed T cells depleted of adherent cells, while they are completely ineffective in the generation of a primary response *in vitro* from adherent cell-depleted, T cell-enriched populations.

4 Discussion

Previous results have shown that insertion of SV glycoproteins into the target cell membrane is required for effective lysis by CTL [1, 2, 21]. Having investigated the requirements for the primary and secondary induction of cytotoxic virus-specific T lymphocytes *in vitro* [10], we now studied the generation of CTL by SV preparations of different functional activities. We have used three preparations of noninfectious virus differing with respect to their capacity to interact with cells: (a) intact virions with virus-cell fusion capacity (β-PL-SV), (b) trypsin-treated virions with cell-binding, but no fusion capacity (Try-SV(F^-)) and (c) V8 protease-treated virions lacking cell-fusion or cell-binding capacity (V8-SA-SV($HANA^-$)). When virus preparations were added to the culture, which were active (β-PL-SV) or inactive in fusion (Try-SV(F^-)), they could trigger T cells to mount a cytotoxic response even after depletion of phagocytic and adherent cells. However, virus preparations without the capacity to attach to cells (V8-SA-SV($HANA^-$)) [22–24] lacked the capacity to induce CTL unless macrophages were present in the culture. β-PL-SV and Try-SV(F^-), presented on tumor stimulator cells, were effective in activating antigen-primed cells after depletion of adherent cells, while adherent cell-depleted unprimed responder cell populations failed to respond.

The fact that the noninfectious SV preparation (β-PL-SV) can be used to activate CTL *in vitro* and *in vivo* could be explained by its virus-cell fusion capacity. This mechanism provides integration of envelope proteins into cell membranes creating a situation qualitatively similar to cell infection by which the nascent antigens are inserted by fusion from within into the cell membrane. This result is in accord with the hypothesis [9, 12] that activation of H-2-restricted CTL strictly requires copresentation of H-2 and the associative neoantigen (in this case a viral glycoprotein) as integral membrane components of one cell. The hypothesis requires that (a) only antigens which fuse actively with cells or (b) only antigens that are actively taken up by cells which are active in presentation [25] should allow killer cell generation. This would explain the observations that noninfectious viruses which do not fuse with cells, can induce virus-specific CTL *in vivo* [3–5] or *in vitro* in the presence of macrophages [8].

The mechanism of *in vivo* presentation of noninfectious viral antigens or nonfusing viruses for triggering of the precursors to the CTL is difficult to investigate. Published data derived from *in vitro* stimulation of secondary CTL responses indicate that noninfectious virus and viral protein can trigger a secondary response [6, 8, 11, 12]. It has been argued that the primary response *in vivo* and the secondary response *in vitro* have qualitatively and quantitatively different requirements for antigen presentation [12]. We have no direct information as to the mechanisms of CTL generation *in vivo* with inactivated SV. The argument that the primary and secondary anti-viral CTL response differ mainly quantitatively because different numbers of virions are integrated into cell membranes [12] seems not to apply to the SV model. First, on the basis of earlier calculations on the quantitative requirements for target cell formation [1, 13] and present data on CTL induction, we conclude that both events require similar amounts of viral protein. These protein requirements are not substantially changed by the inactivation of the fusion capacity. Second, differences seen between the primary and secondary CTL response to P815 stimulator cells carrying viral antigens were not due to surface antigen density.

Yet, *in vitro* activation of antigen-primed cells and *in vitro* sensitization of unprimed cells differ qualitatively. Addition of the virus preparations had shown that all three were active, but the activation was differentially susceptible to adherent cell depletion techniques. When we correlated the requirements for adherent cells during CTL induction with the cell interaction capacity of the virus preparations, those virions with reduced ability to interact actively with cells were found to be more dependent on the presence of adherent cells in order to act as antigen. So the V8-SA-SV ($HANA^-$) preparation without cell-binding activity [22–24, 26], although capable of inducing CTL generation, strictly required the presence of adherent cells in order to generate a primary response. The experiments of Zinkernagel et al. [27] suggested a crucial role for macrophages in the selection of H-2 specificity of the immune response. Our data similarly suggest a function of macrophages or antigen-presenting cells during primary generation of CTL.

This role of adherent cells was even more prominent when the antigen was presented on tumor cells. The interpretation of the tumor stimulator cell experiments are somewhat limited by the various possible ways in which the viral antigens could have been presented on other cells in the culture. Unfortunately, we could not use fixed cells since there is no primary response *in vitro* to this type of stimulator cells [10]. Still, the results obtained so far show that the generation of a primary response, using tumor stimulator cells, is susceptible to adherent cell depletion even when the viral antigens are integrated into the cell membrane. The P815 cells lack antigens coded for by the I region [28], and further experiments are in progress to investigate this point. The data indicate that processing by antigen-presenting cells cannot be completely explained merely by integration of antigens into the membrane of cells carrying syngeneic H-2K and H-2D region products.

In spite of the difficulties in interpreting processing during sensitization, we were able to compare the requirements of *in vitro* activation of antigen-primed T cells with the conditions which allow aggressive interaction of effector-to-target cells. Antigen-primed cells were triggered to generate CTL after addition of the three preparations to the cell culture, even after depletion of adherent and phagocytic cells. These conditions were not different when β-PL-SV and Try-SV(F^-) were bound to (P815) tumor stimulator cells. The same conditions, however, *i.e.* binding of Try-SV(F^-) to P815 cells, are insufficient to allow target cell formation, even when a high amount of virus is used under long-term incubation conditions. In summary, there is evidence of differences of antigen presentation for primary sensitization and activation of antigen-primed CTL *in vitro*. Furthermore, reactivation is possible with cell-bound antigens and does not require presentation of viral antigens as integral membrane components.

Although in previous experiments, we were able to prepare targets with preparations containing only the two viral glycoproteins devoid of the matrix protein [13], we could not further subdivide the CTL populations with specificity for HANA or F, respectively. There is strong evidence that the fusion activity is involved in target cell formation, but this does not implicate a function of F as a target antigen. Because of the lack of target cells carrying either the HANA or the F protein alone, integrated into the cell membrane, we were not able to study this question at the effector cell stage. However, our results show that virions devoid of the HANA protein will induce *in vitro* generation of primary and secondary specific CTL, thus providing evidence of a T cell subpopulation which can be specifically triggered by the F protein. On the other hand, the experiments with Try-SV (F^-) indicate the existence of a T cell subpopulation with specificity for the HANA protein of SV. The latter experiment needs qualification. Although the functional activity of F had been destroyed on these virions, the cleaved F glycoprotein is not detached from the virus particle. We are not able to exclude the possibility that the functionally inactivated cleaved protein is still antigenically active. Until experiments have been carried out using virion subunit preparations containing HANA protein exclusively, the experimental evidence of HANA-specific CTL must be considered as preliminary.

We thank Drs. B. Kindred, V. Schirrmacher and M. Simon for helpful criticisms. The able technical assistance was provided by Ms. I. Neuschäfer-Rube, and we are indebted to Ms. U. Rohmann for patient typing assistance.

Received March 14, 1979; in revised form July 4, 1979.

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