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Immunology

Antigen–liposome modification of target cells as a method to alter their susceptibility to lysis by cytotoxic T lymphocytes

(Sendai virus/liposomes/major histocompatibility complex/H-2 restriction)

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ABSTRACT **A method of liposome modification of cell surfaces to render unsuitable target cells susceptible to lysis by anti-viral cytotoxic T lymphocytes (CTLs) is described. Liposomes containing the hemagglutinin–neuraminidase (HN) and fusion (F) glycoproteins of Sendai virus as well as purified H-2K^k antigens were capable of binding to the surface of H-2-negative cells and rendering those cells susceptible to lysis by B10·A anti-Sendai virus or anti-H-2K^k CTLs. The absence from the modifying liposomes of the HN or F proteins or H-2K^k antigens eliminated the ability of the target cells to be recognized and lysed by either effector cell population. Vesicles containing HN, H-2K^k molecules, and inactive fusion protein (Fo) were not capable of increasing the susceptibility of H-2-negative target cells to lysis. Liposomes containing inactive fusion protein were similarly unable to render H-2-positive target cells susceptible to lysis by anti-Sendai virus CTLs, suggesting that fusion of the liposomes to the cell surface is a prerequisite to lysis. It did not appear that attachment of liposomes to the cell surface was sufficient for generation of susceptible targets, however, because attachment to the cell surface was observed, as long as the HN glycoprotein was present in the liposomes. These results indicate that purified H-2K^k glycoproteins are target antigens for anti-H-2^k CTLs and that B10·A anti-Sendai virus CTLs recognize in an H-2-restricted manner the HN, F, or both glycoproteins of Sendai virus in the context of the purified H-2K^k glycoproteins. This technique of liposome modification of cell surfaces has potential applications in the examination of CTL antigen recognition and immunotherapy of many viral and neoplastic diseases.**

Recognition and lysis of cells with newly acquired foreign antigens by cytotoxic thymus-derived lymphocytes (CTLs) is probably a major mechanism for host resistance to viral infections. The specific recognition of these neoantigens requires the functional interaction of the CTLs with both the foreign antigen of interest and target cell antigens encoded by the major histocompatibility complex (MHC; *H-2* in the mouse) (1–3). One of the primary difficulties in the study of these interactions has been an inability to identify those specific viral or MHC molecules recognized by CTLs.

Recent evidence from our laboratory has suggested that reconstituted Sendai virus glycoproteins [hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins] incorporated into model phospholipid vesicles are capable of fusing with cells and rendering them susceptible to recognition by anti-Sendai virus CTLs (4). In this paper we describe the modification of H-2-negative target cells by fusion with phospholipid vesicles containing Sendai virus glycoproteins HN and F and purified H-2K^k glycoproteins, and we describe the subsequent acquisition of susceptibility of the target cells to lysis by anti-H-2K and B10·A anti-Sendai virus CTLs. This method has potential application both for the identification of the specific neoantigen and H-2 moieties recognized by CTLs and for modification of nonimmunogenic target cells for immunotherapeutic purposes.

MATERIALS AND METHODS

Mice. Male and female mice of the following strains were used: BALB/c (*d,d*), BALB·HTG (*d,b*), BALB·K (*k,k*), and B10·A (*k,d*). These were either purchased from Cumberland View Farms (Clinton, TN) or produced in our own breeding colony from stock obtained from Herman Eisen (Massachusetts Institute of Technology). Letters in parentheses indicate the *H-2K* and *H-2D* alleles.

Virus. Sendai virus with the inactive fusion glycoprotein (Fo) was grown in Madin–Darby bovine kidney (MDBK) cells, the virus was purified, and Fo was activated by treatment with trypsin (5 μg/ml, TPCK-trypsin; Worthington) as described by Scheid and Choppin (5). Viral infectivity was inactivated by ultraviolet light (UV) as described (6, 7). In the studies reported here only UV-inactivated Sendai virus was used to elicit anti-Sendai virus CTLs or to render cells susceptible to lysis by anti-Sendai virus CTLs (6, 7).

Antisera. Alloantiserum to H-2^k was prepared by multiple intraperitoneal injections of 2.0×10^7 BALB·K spleen cells into BALB/c mice (8). Monoclonal anti-H-2K^k was obtained from Becton Dickinson FACS Systems (Mountain View, CA) (9).

Rabbit anti-Sendai virus serum was made by multiple intradermal injections of purified Sendai virus (4). Rabbits were injected once with 100 μg of purified Sendai virus in complete Freund's adjuvant and four times (3- to 4-week intervals) with 25 μg of Sendai virus in incomplete adjuvant. Anti-Sendai virus antibodies were evident after the fifth injection and were assayed by the capacity to precipitate HN and F glycoproteins.

Purification of Sendai Virus and H-2K^k Glycoproteins. The glycoproteins of MDBK-grown Sendai virus with and without trypsin treatment were isolated by extraction of the purified virus with Triton X-100 (final concentration of 2.0%, vol/vol) as described by Scheid and Choppin (5). The HNF, HNFo, HN, and F glycoproteins were purified as described by Hale *et al.* (4). H-2K^k was purified from YAC cells (*k,d*) as described by Herrmann and Mescher (10). H-2^k molecules were eluted from Sepharose 4B columns conjugated with monoclonal anti-H-2K^k antiserum (clone 11-4.1, Becton Dickinson), with 0.2% sodium deoxycholate in 140 mM NaCl.

Purification of HNF or HNFo and H-2K^k was monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis (11) and au-

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Abbreviations: CTLs, cytotoxic thymus-derived lymphocytes; F, fusion glycoprotein of Sendai virus; Fo, inactive fusion protein of Sendai virus; HN, hemagglutinin–neuraminidase glycoprotein of Sendai virus; MDBK, Madin–Darby bovine kidney cells; MHC, major histocompatibility complex.

toradiography. Tumor cells or purified Sendai virus were iodinated with ^{125}I in the presence of lactoperoxidase and H_2O_2 (12). The cells or virus were washed by centrifugation and each protein was purified as described above. Samples were taken, subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (8), and identified by autoradiography.

Media. RPMI 1640 medium (GIBCO) was supplemented with 10% fetal calf serum (Flow Laboratories; heat-inactivated at 56°C for 45 min), 0.03% glutamine, and penicillin and streptomycin (Flow Laboratories, McLean, VA) (13).

Cell Lines. The C58 (*k,k*) cultured lymphoma lines $R1^+$ and $R1^-$ were generously provided by H. Eisen. Both tumors were grown in supplemented RPMI 1640 medium. The $R1^-$ variant was originally obtained by selection among $R1^+$ cells for variants that lacked β_2-microglobulin; the $R1^-$ variant lacks *H-2K* and *H-2D* products, and also the TL antigen (14). Lymphoma line YAC (*k,d*) was used as the cell source for $H\text{-}2K^k$ glycoproteins.

Production of CTLs. Secondary anti-Sendai virus CTLs were elicited by using responder spleen cells from mice primed 4–6 weeks earlier by intraperitoneal injection with 100 μg of UV-inactivated Sendai virus [protein determination by the method of Lowry *et al.* (13)]. Nonirradiated syngeneic spleen cells (3.0×10^6; erythrocytes removed with NH_4Cl) were preincubated as described (4, 5) with 30 μg of UV-inactivated Sendai virus [Sendai protein determined by the method of Lowry *et al.* (13)]. Suspensions of Sendai virus-primed spleen cells were treated with NH_4Cl to remove erythrocytes (15). Then 7.0×10^6 Sendai virus-primed responder spleen cells were incubated with 3.0×10^6 Sendai virus-coated, nonirradiated, syngeneic spleen cells for 5 days in 2.0 ml of supplemented RPMI 1640 medium with 2-mercaptoethanol (50 μM) in 1.7×1.6 cm wells (Linbro) in an atmosphere of 6% CO_2/94% air.

To produce anti-H-2 CTLs, spleen cells were harvested from the mice of the appropriate strain, washed by centrifugation, and counted. To 7.0×10^6 viable spleen cells in 1.7×1.6 cm wells were added 3.0×10^6 x-irradiated [2000 roentgens (0.5 coulomb/kg) from a Picker instrument] allogeneic spleen cells in a total volume of 2.0 ml of supplemented RPMI 1640 medium. After mixing, cell suspensions were incubated at 37°C in 94% air/6% CO_2. Killing assays were performed on day 5.

Reconstitution of Viral and H-2 Antigens into Liposomes. In a typical preparation, 200 μg of egg lecithin plus cholesterol [30% (wt/wt); Calbiochem] dissolved in chloroform was dried in the form of a film under N_2. This film was then dissolved into a solution containing 0.2% sodium deoxycholate, purified $H\text{-}2K^k$ antigens, and Sendai virus glycoproteins at a lipid-to-protein wt ratio of 1:1 in a final volume of 4 ml. The protein ratio (wt/wt) of H-2 to HNF or HNFo (or H-2 to isolated HN or F) was 1:1. This solution was then dialyzed for 36–48 hr against phosphate-buffered saline at 4°C. The dialysate was recovered as an opalescent solution with greater than 80% of the protein associated with the vesicles (4, 16, 17).

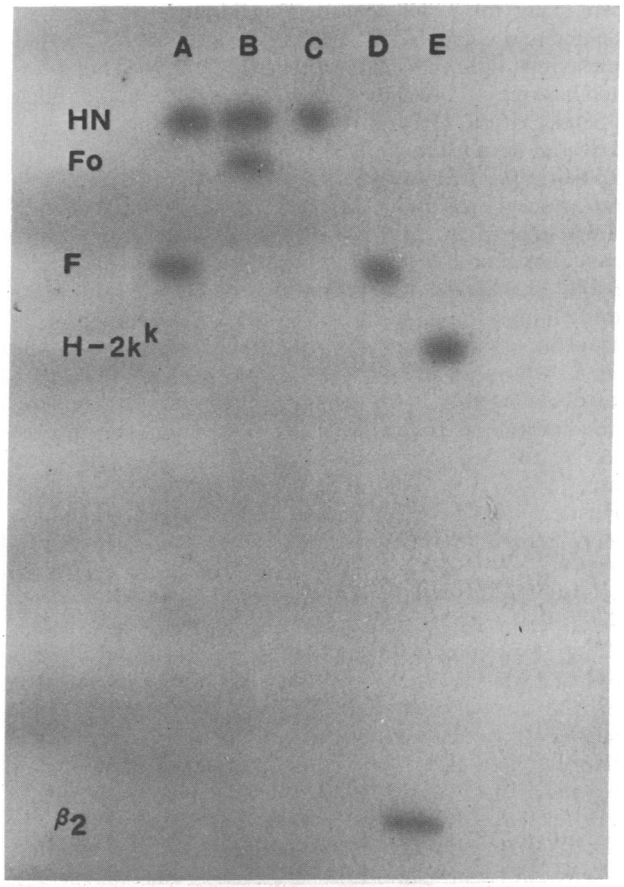

FIG. 1. Electrophoretic analysis of purified viral and $H\text{-}2K^k$ glycoproteins: viral glycoproteins and $H\text{-}2K^k$ were incorporated into liposomes, analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, and identified by autoradiography. Lane A, HNF; lane B, HNFo; lane C, HN; lane D, F; lane E, $H\text{-}2K^k$. β_2, β_2-microglobulin.

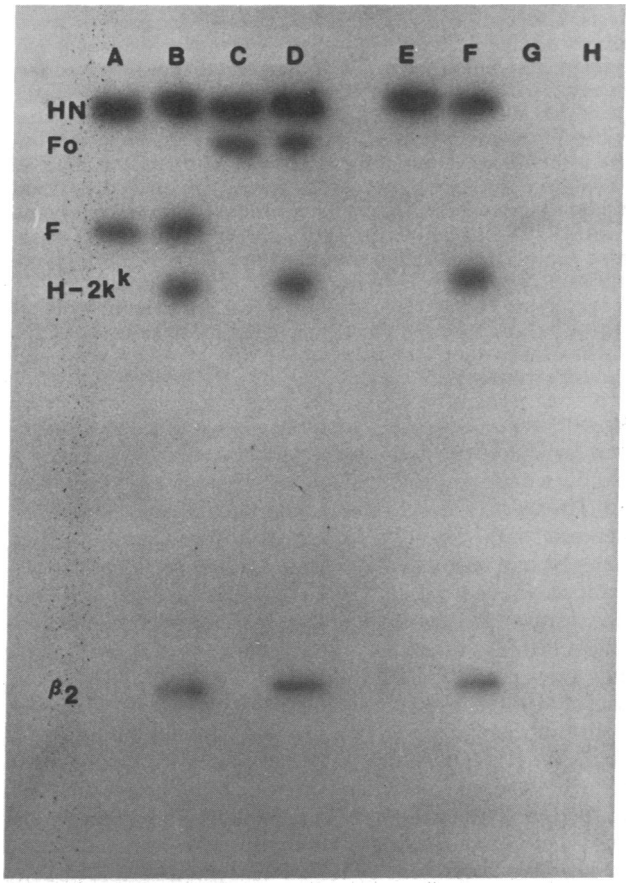

FIG. 2. Evaluation of the ability of different liposomes containing ^{125}I-labeled viral and $H\text{-}2K^k$ glycoproteins to bind to 1.0×10^4 $R1^-$ cells. Total protein for all liposomes tested was 10 μg per 10^4 cells. The cells were incubated with the liposomes, washed by centrifugation ($450 \times g$, 5 min), solubilized in electrophoresis sample buffer, and subjected to electrophoresis on a $NaDodSO_4$/polyacrylamide gel, and the labeled proteins were identified by autoradiography. Liposomes containing HNF (lane A), HNF-$H\text{-}2K^k$ (lane B), HNFo (lane C), HNFo-$H\text{-}2K^k$ (lane D), HN (lane E), HN-$H\text{-}2K^k$ (lane F), F (lane G), and F-$H\text{-}2K^k$ (lane H) were analyzed for their ability to bind to $R1^-$ cells. β_2, β_2-microglobulin.

Target Cells. $R1^-$ target cells (1.0×10^6) were labeled with 200 μCi (7.4 megabecquerels) of $Na_2{}^{51}CrO_4$ in 200 μl of 0.15 M NaCl for 20 min at 37°C. This suspension was then diluted 1:3 with supplemented RPMI 1640 medium and incubated for another 60 min at 37°C in an atmosphere of 94% air/6% CO_2. ^{51}Cr-Labeled $R1^-$ cells (1.0×10^5) were rendered susceptible to lysis by CTLs by incubation with reconstituted viral and H-2K^k liposomes (100 μg of protein) in RPMI 1640 medium for 15 min at 4°C and 30 min at 37°C. The cells were then washed by centrifugation at 450 × *g* and suspended with effector cells in a total volume of 100 μl of supplemented RPMI 1640 medium.

Cytotoxicity Assays. ^{51}Cr-Labeled target cells (1.0×10^3) were mixed with serial dilutions of effector spleen cells in a total volume of 200 μl of supplemented RPMI 1640 medium. This mixture was incubated 4–6 hr at 37°C in an atmosphere of 6% CO_2/94% air. The assay was stopped by diluting with cold phosphate-buffered saline and the amounts of radioactivity in the supernatant and pellet were determined (13). Percent specific release was calculated as $100[(E - C)/(1 - C)]$, in which E is the fraction of ^{51}Cr released by antigen-stimulated effector cells and C is the fraction of ^{51}Cr released by a mock-stimulated responder population. Percent release was calculated as $100(E/T)$, in which E is the amount of radioactivity released by target cells incubated with antigen-stimulated effector cells and T the total amount of radioactivity in each assay tube.

RESULTS

An analysis of the purity of the isolated HNF, HNFo, HN, F, and H-2K^k glycoproteins incorporated into phospholipid vesicles is shown in Fig. 1. Analysis of these liposomes by NaDodSO$_4$/polyacrylamide gel electrophoresis demonstrated that HNF (HN, 69,000 daltons; F, 56,000 daltons), HNFo (Fo, 65,000 daltons), HN, F, and the H-2K^k (heavy chain 45,000 and light chain 12,000 daltons) molecules were incorporated into liposomes and that these molecules were pure.

In order to evaluate the ability of liposomes containing ^{125}I-labeled H-2K^k, HNF, or HNFo molecules to associate with cells, 1.0×10^4 $R1^-$ cells were incubated with liposomes of various compositions (total protein per 10^4 cells = 10 μg). After incubation at 37°C for 1.0 hr the cells were washed twice by centrifugation at 450 × *g* for 5 min and prepared for analysis by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 2). The results indicated that phospholipid vesicles containing HN became associated with $R1^-$ cells. However, vesicles with only H-2K^k or F were relatively inefficient in binding to the $R1^-$ cell surface. This is consistent with HN being the major receptor binding protein of Sendai virus (5).

The susceptibility of liposome-modified, ^{51}Cr-labeled $R1^-$ cells to lysis by B10·D2 anti-B10·A CTLs and B10·A anti-Sendai virus CTLs is shown in Table 1. BALB/c anti-Sendai virus CTLs or B10·A anti-B10·D2 CTLs, however, were unable to lyse the $R1^-$-HNF-H-2K^k target cell (Table 1). Therefore, these modified target cells were recognized in an H-2 restricted manner. This result suggests that the HNF-H-2K^k molecules were incorporated in a functional manner in the membranes of the $R1^-$ cells. The results indicated that only those $R1^-$ cells incubated with HNF and H-2K^k-containing liposomes were lysed by either of the effector populations. The absence of HN, F, or H-2K^k in the modifying liposomes eliminated the ability of the target cells to be recognized and lysed. The failure of the HNFo-H-2K^k vesicles to render target cells susceptible to lysis suggests that an active fusion protein is required. An important control in these experiments shows that HNF and H-2K^k incorporated into separate liposomes ($R1^-$-HNF + H-2K^k, Table 1) are ineffective at rendering $R1^-$ cells susceptible to lysis.

$R1^+$ cells, which possess functional H-2K^k, do not require modification by H-2-containing liposomes to be recognized by CTLs. HNF- but not HN-, F-, or HNFo-containing liposomes were able to render $R1^+$ cells susceptible to lysis by BALB·K anti-Sendai virus CTLs (Table 2). BALB/c anti-Sendai virus CTLs were unable to lyse the HNF-modified $R1^+$ cells. The H-2 restriction exhibited suggests that HNF was able to associate with the *H-2K* and *H-2D* gene products of the cell in a functional manner.

Table 1. Susceptibility of liposome-modified $R1^-$ cells to lysis by CTLs

Effector cells	E:T ratio	^{51}Cr release from targets, %										
		$R1^-$	$R1^+$	$R1^-$-HNF-H-2K^k	$R1^-$-HNF + H-2K^k	$R1^-$-HN-H-2K^k	$R1^-$-F-H-2K^k	$R1^-$-HNFo-H-2K^k	$R1^-$-SV	$R1^+$-SV	P815	P815-SV
B10·D2 anti-B10·A	100:1	13.7	62.7	48.7	7.4	8.0	9.9	8.2	9.4	47.3	8.2	10.1
	50:1	16.2	31.7	27.9	8.2	8.6	12.4	14.2	8.8	38.6	9.2	9.8
	25:1	11.8	21.6	10.9	9.7	9.2	11.3	11.7	8.4	19.2	9.4	11.2
B10·A anti-B10·D2	100:1	15.6	10.4	6.7	11.2	11.7	9.2	6.2	8.1	8.6	60.7	57.7
	50:1	11.7	11.2	9.4	10.4	8.2	8.4	8.1	7.9	8.4	22.7	42.8
	25:1	10.4	13.4	9.7	9.6	7.4	6.2	7.2	9.8	6.2	11.8	21.7
B10·A anti-Sendai virus	100:1	11.3	11.9	42.8	11.2	8.4	7.9	14.3	8.7	72.7	8.2	22.3
	50:1	11.2	6.7	31.8	6.7	7.2	9.7	10.2	8.3	62.4	9.2	19.8
	25:1	10.8	14.8	22.7	9.7	8.3	8.2	11.4	7.7	45.7	8.7	16.2
BALB/c anti-Sendai virus	100:1	10.4	11.2	9.8	10.4	10.2	9.8	3.8	6.2	11.7	9.2	62.7
	50:1	14.3	11.4	7.9	9.7	8.5	11.2	11.7	8.1	10.7	9.3	32.8
	25:1	11.7	6.7	6.9	8.9	7.9	14.2	9.7	9.8	9.7	8.7	21.7
B10·A normal spleen	100:1	9.7	11.7	6.2	11.8	9.7	8.3	12.2	13.1	9.8	6.7	11.2
	50:1	8.3	12.0	8.3	10.7	9.6	11.2	8.7	10.4	6.2	8.4	10.3
	25:1	6.4	7.5	9.2	10.3	8.9	16.4	9.2	9.7	7.7	6.9	9.7

Effector cells were incubated with 10^3 ^{51}Cr-labeled targets for 6 hr at 37°C. E:T, effector to target cell ratio. ^{51}Cr-Labeled $R1^-$ cells were modified with liposomes containing 100 μg of HNF and H-2K^k incorporated into the same liposome ($R1^-$-NHF-H-2K^k), HNF, and H-2K^k incorporated into separate liposomes ($R1^-$-HNF + H-2K^k), purified HN and H-2K^k in the same liposomes ($R1^-$-HN-H-2K^k), purified F and H-2K^k in the same liposomes ($R1^-$-F-H-2K^k), or HNFo and H-2K^k incorporated in the same liposomes (HNFo-H-2K^k). Viral glycoproteins and H-2K^k molecules were always at a ratio of 1:1 (wt/wt) whether in the same or separate liposomes. $R1^-$, $R1^+$, and P815 cells were modified with UV-inactivated Sendai virus (SV; 10 μg of virus per 10^6 cells) and used as controls. Each value represents the average of six measurements with the standard deviations for any of the determinations never exceeding 4.7%. Significant lysis (Student's t test, $P < 0.01$) is indicated by underlined values.

Table 2. Susceptibility of liposome-modified R1$^+$ cells to lysis by CTLs

Effector cells	E:T ratio	^{51}Cr release from targets, %										
		R1$^-$	R1$^+$	R1$^-$-SV	R1$^+$-SV	R1$^+$-HNF	R1$^-$-HNF	R1$^+$-HN	R1$^+$-F	R1$^+$-HNFo	P815	P815-SV
BALB/c anti-BALB-K	100:1	12.7	56.3	11.2	61.7	57.3	11.7	42.7	47.9	59.2	8.7	9.2
	50:1	13.2	32.8	13.8	42.8	42.3	9.8	28.2	38.4	48.2	9.2	10.3
	25:1	9.8	27.5	12.7	32.8	28.7	8.2	3.5	7.8	20.1	11.3	10.7
BALB-K anti-BALB/c	100:1	6.7	11.2	12.6	13.2	8.7	6.5	6.8	9.2	8.9	72.3	57.0
	50:1	10.4	10.7	13.2	9.6	8.7	8.2	15.3	8.4	7.6	56.7	48.3
	25:1	8.2	5.8	9.6	7.8	9.2	5.2	9.8	6.7	9.1	17.9	29.0
BALB-K anti-Sendai virus	100:1	9.2	16.2	8.2	47.9	31.2	11.9	9.7	8.4	7.2	9.1	6.2
	50:1	9.3	13.4	7.2	41.2	27.2	/	11.7	8.2	8.5	8.2	3.4
	25:1	10.1	12.8	7.6	36.5	24.7	12.2	16.4	9.3	8.7	10.3	8.2
BALB/c anti-Sendai virus	100:1	14.8	12.3	8.3	15.8	15.8	12.8	11.8	10.4	10.2	8.7	55.4
	50:1	15.2	11.7	8.6	16.2	16.2	11.7	6.2	9.2	9.	9.3	43.2
	25:1	16.2	8.2	9.2	13.4	13.1	16.4	8.6	9.7	10.5	8.4	26.2
Normal BALB-K spleen cells	100:1	13.8	13.4	10.4	12.8	12.7	13.4	9.4	9.3	8.3	9.7	6.2
	50:1	14.2	8.7	9.8	14.7	13.6	10.2	11.3	10.6	10.3	9.8	8.4
	25:1	11.7	9.1	6.5	13.2	9.1	9.8	11.7	8.7	9.4	9.2	5.2

Effector cells were incubated with 10^3 ^{51}Cr-labeled target cells for 6.0 hr at 37°C. ^{51}Cr-Labeled R1$^-$ and R1$^+$ cells were modified with liposomes containing 100 μg of HNF, HN, F, or HNFo. R1$^+$, R1$^-$, and P815 cells were also modified with UV-inactivated Sendai virus (10 μg of virus per 10^6 cells) and used as controls. Each value represents the average of six measurements with the standard deviation for any of the determinations never exceeding 4.2%. Abbreviations and conventions as in Table 1.

DISCUSSION

We have described a method by which target cells that are not susceptible to lysis by allogeneic (anti-H-2K^k) or syngeneic (anti-Sendai virus) CTLs can be made susceptible to lysis by these effectors. The method required the incorporation of HNF glycoproteins from Sendai virus into phospholipid vesicles containing purified H-2K^k antigen and the subsequent fusion of these vesicles with the target cell. When F was absent or Fo was present in the modifying liposomes, target cells were not rendered susceptible to lysis. The inability of anti-Sendai virus or anti-H-2K^k CTLs to lyse cells modified with F resulted from the lack of binding of liposomes reconstituted with only F or F-H-2K^k glycoproteins to target cells. However, as long as HN was present within the liposome, attachment of the liposome to the target cell surface was observed. This result is consistent with the idea that HN is the major surface binding protein of the Sendai virus (5). These observations imply that attachment of the vesicle to the target cell surface was necessary but not sufficient for the acquisition of susceptibility to lysis. The fact that HNFo-modified R1$^-$ or R1$^+$ cells were not susceptible to lysis suggests that active fusion of the liposome to the cell surface was an additional prerequisite to lysis. This result is consistent with the observation that Sendai virions with inactive fusion protein do not render target cells susceptible to lysis (4, 18).

For both allogeneic (anti-H-2) and syngeneic (anti-Sendai virus) CTLs, the incorporation of H-2K^k into the HNF-containing liposomes was required for effective modification of the H-2-negative target cells but not for modification of H-2-positive target cells. It would appear, therefore, that allogeneic CTLs require recognition of the 45,000-dalton glycoprotein coded in the *H-2K* region, while anti-viral CTLs require recognition of both the *H-2K* gene product and the viral antigens for effective lysis of target cells. These results indicate that H-2 antigens incorporated into cell membranes by liposome fusion are functional both in the phenomenon of H-2 restriction and in ability to be recognized by allogeneic CTLs.

Recently, Volsky *et al.* (19) reported that the anion transport protein from erythrocyte membranes could be incorporated into leukemic cells through the use of liposomes containing Sendai virus glycoproteins and the transport protein. We have confirmed this technique and shown it to be applicable to analysis of the antigenic specificity of CTLs. These techniques are potentially important in the development of immunotherapeutic measures to control many viral and neoplastic diseases.

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