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Immunology

"E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens

(cytotoxic T cells/histocompatibility HLA-H-2 antigens/terminal glycosylation/viral glycoprotein)

HANS-GERHARD BURGERT*, JANET L. MARYANSKI†, AND SUNE KVIST*‡

*Swiss Institute for Experimental Cancer Research; and †The Ludwig Institute for Cancer Research, Lausanne Branch, Ch. des Boveresses 155, CH-1066 Epalinges s/Lausanne, Switzerland

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ABSTRACT **The E3 19,000-dalton protein termed "E3/19K" of adenovirus type 2 binds to human class I histocompatibility antigens (HLA antigens). Human 293.12 cultured cells that express a cloned gene for the E3/19K protein show reduced levels of HLA antigens on the cell surface compared to parental 293 cells. We have transfected these cell lines with plasmid DNA containing the murine histocompatibility *H-2K*^{d} allele to demonstrate that this antigen binds also to the E3/19K protein. The resulting association prevents the H-2K^d antigen from being terminally glycosylated and inhibits its cell-surface expression. Two murine cytolytic T-lymphocyte clones specific for HLA antigens and restricted by the H-2K^d antigen lyse the human 293K^d cells. In the presence of the E3/19K protein, a dramatically reduced cell surface density of both HLA and H-2K^d antigens was shown. This decreased amount of cell-surface HLA/H-2K^d antigens correlated with a reduction in susceptibility to lysis of the target cells. In particular, the cell-surface level of the H-2K^d antigen, which is the restricting element, was crucial for efficient lysis. Thus, the E3/19K protein of adenovirus type 2 indirectly reduces the cellular immune recognition in the *in vitro* system. This might be the mechanism involved in latent and persistent infections caused by adenoviruses *in vivo*.**

The major histocompatibility complex (MHC) encodes a variety of proteins involved in different immunological phenomena. The class I antigens of both mouse (H-2 antigens) and human (HLA antigens) are cell-surface integrated molecules with a M_r of ≈45,000. They are noncovalently associated with β_2-microglobulin (M_r, 12,000; refs. 1–3). One major function of these antigens is to condition T cells to become cytotoxic T lymphocytes (CTL) during virus infections. This is likely to occur by altering the class I antigens and is induced by interaction with foreign (viral) proteins. The cytotoxic T cells can precisely distinguish between the infected and noninfected cells. This T-cell recognition is known as H-2 or HLA antigen (MHC) restriction (4). Thus, a qualitative (phenotypic) change in the histocompatibility class I antigen is of crucial importance for T-cell recognition. Furthermore, a quantitative variation in the amounts of HLA/H-2 antigens expressed on the cell surface might influence the T-cell response.

Several investigators have demonstrated that the E3 19,000-dalton protein termed "E3/19K" of adenovirus type 2 (Ad2) binds to and forms a molecular complex with class I HLA/H-2 antigens (5–8). Recently, we have shown that the formation of the HLA–E3/19K complex prevents the HLA antigens from being correctly processed by inhibiting their terminal glycosylation (9). More importantly, the HLA–E3/19K association reduces the cell-surface expression of HLA antigens. We now address the question of whether or not this reduced level of class I antigens influences the cytotoxic T-cell response. If so, our results might show a possible molecular mechanism whereby adenoviruses and other viruses escape the cellular immune response.

For obvious reasons the T-cell response against adenovirus-infected cells cannot be studied *in vivo* in humans. Therefore, we have used an *in vitro* system in which the murine H-2K^d antigen functions as the restriction element, and endogenous HLA molecules function as the "foreign" antigens (10). This would allow us to use murine T-cell clones in our analysis. In this paper we demonstrate that a decreased level of the restricting element on the cell surface dramatically influences the ability of cytotoxic T cells to recognize and kill these cells. Thus, the complex formation between the histocompatibility class I antigen and the E3/19K protein of Ad2 indirectly reduces the cytotoxic T-cell response.

MATERIALS AND METHODS

Cell Culture and DNA Transfection of Cells. The human cell line 293 was established by transformation of embryonic kidney cells with Ad5 (11). The 293.12 cell line is a derivative of 293 cells transfected with the *Eco*RI D fragment of Ad2 (9). The establishment of the thymidine kinase negative (TK$^-$) mouse mastocytoma cell line P815 (HTR) that can be transfected efficiently has been described (12). Transfection of cells with plasmid DNA was done essentially as described previously (13). Two selection systems were used: (*i*) the neomycin phosphotransferase gene conferring neomycin resistance (*neo*; phenotype Neo^R) together with the drug G-418 (14) and (*ii*) the gene (*hyg*) conferring hygromycin-resistance (Hyg^R; refs. 15 and 16) with hygromycin B (17). 293 cells were transfected with *neo* and the plasmid containing the murine *H-2K*^{d} allele (18). For the 293.12 cells, which are already *neo*-positive, the *hyg* system was used. The concentrations of G418 and hygromycin were 560 and 200 μg/ml, respectively.

Antibodies, Cell Labeling, Immunoprecipitation, and Na-DodSO$_4$/PAGE. Two different monoclonal antibodies against the H-2K^d antigen were used: 34-1-2s (19) and 20-8-4s (20). The antibodies against HLA-A, -B, and -C antigens were W6/32 (21) and B9.12.1 (22). The former antibody detects HLA antigens when they are combined with human β_2-microglobulin, whereas the latter reacts with HLA associated with both human and mouse β_2-microglobulin.

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Abbreviations: Ad1, Ad2, Ad5, and Ad6, adenovirus types 1, 2, 5, and 6; CTL, cytolytic T lymphocytes; FACS, fluorescence-activated cell sorter; TK, thymidine kinase; MHC, major histocompatibility complex.

‡To whom reprint requests should be addressed.

Cells were labeled as described (9), and immunoprecipitation and NaDodSO$_4$/PAGE were carried out as described (23).

Flow Cytometric Analysis and Cytolytic Assay. The details for the fluorescence-activated cell sorter (FACS) analysis have been described (9). The cells were analyzed with a FACS II instrument. The cytolytic assay using ^{51}Cr-labeled target cells (2000 cells per well) at various effector-to-target ratios was terminated after 4 hr at 37°C. Two different CTL clones (anti-A24 clones 10 and 12) were used and have been described (10). The percentage of specific lysis was calculated by the formula: (experimental − spontaneous release/maximum − spontaneous release) × 100.

RESULTS

Expression and Association of the Mouse H-2K^d Antigen with the E3/19K Protein of Ad2 in Human Cells. It was of interest to express the H-2K^d antigen in human cells for at least two reasons. First, we determined whether or not mouse class I H-2 antigens associate with the E3/19K protein in human cells. Secondly, we would like to use human cells expressing the E3/19K protein and the H-2K^d antigen as target cells for HLA-specific, H-2K^d-restricted CTL clones.

The human cell line 293 (9, 11) was transfected with plasmid DNA containing the *H-2K^d* gene (18). The neomycin phosphotransferase gene was cointroduced as a selectable marker (14), and cell clones resistant to the drug G418 were selected and analyzed for expressed H-2K^d antigen. Cells from several clones (designated 293K^d) were metabolically labeled with [^{35}S]methionine, and cell lysates were subjected to sequential immunoprecipitation with monoclonal antibodies directed against both H-2K^d and HLA antigens. The precipitates were analyzed by NaDodSO$_4$/PAGE. The antibody against the H-2K^d antigen precipitated a band with a M_r of 46,000 corresponding to the authentic H-2K^d antigen in three clones (Fig. 1, lanes 1, 3, and 5). The antibody against the HLA antigens precipitated two distinct HLA protein bands ($M_r \approx$ 42,000; Fig. 1, lanes 2, 4, and 6). In addition, β_2-microglobulin was present in all six lanes.

We conclude that the introduced *H-2K^d* gene was expressed in human 293 cells in approximately the same amount as the endogenous HLA antigens. Furthermore, the H-2K^d antigen binds human β_2-microglobulin, which is a prerequisite for its cell-surface expression (24).

We next examined if the H-2K^d antigen could associate with the E3/19K protein of Ad-2. In the same way as described above, we introduced the *H-2K^d* gene into 293.12 cells, but instead of the *neo*/G418 system, we used *hyg* as the selectable marker. This cell line previously had been transfected with the *neo* gene and the Ad2 *Eco*RI D fragment (25) and constitutively expresses the E3/19K protein (9).

Several cell clones were labeled with [^{35}S]methionine and analyzed by sequential immunoprecipitation with three different antibodies, anti-H-2K^d, anti-HLA, and anti-E3/19K. The antibody against the H-2K^d antigen precipitated the E3/19K protein in addition to the H-2K^d molecule and β_2-microglobulin. The results with three cell clones (designated 293.12K^d) are shown in Fig. 1 (lanes 7, 10, and 13). The antibody against HLA antigens detected complexes of HLA–E3/19K (lanes 8, 11, and 14), but the E3/19K band was weaker than that obtained with the anti-H-2K^d antibody (compare lanes 7 and 8, 10 and 11, and 13 and 14). The antiserum against the E3/19K protein precipitated the E3/19K protein but did not coprecipitate any further complexes containing H-2K^d or HLA antigens (lanes 9, 12, and 15). Thus, both the mouse H-2K^d and human HLA antigens associate efficiently with the E3/19K protein in human 293.12 cells. Furthermore, a considerable amount of the viral protein is still accessible to the anti-E3/19K antiserum after anti-H-2K^d and anti-HLA precipitations. This overproduction of the E3/19K protein may facilitate an increased efficiency of inhibition of MHC class I proteins on the cell surface.

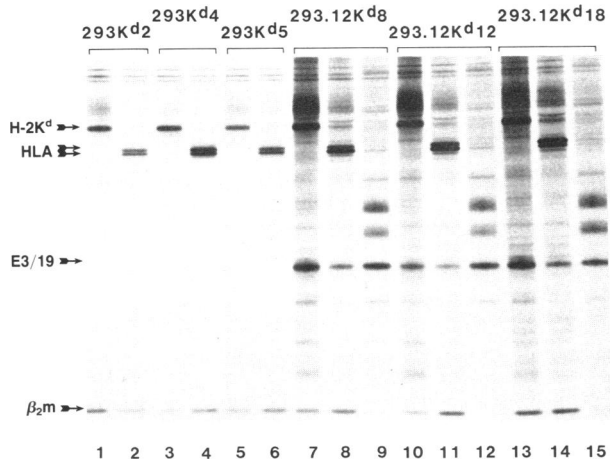

FIG. 1. Immunoprecipitations and NaDodSO$_4$/PAGE of H-2K^d and HLA antigens and E3/19K protein from 293K^d and 293.12K^d clone cells. Cells from the clones indicated in the figure were labeled with [^{35}S]methionine for 40 min and subjected to immunoprecipitation with monoclonal antibodies against the H-2K^d antigen (34-1-2s; lanes 1, 3, 5, 7, 10, and 13) and HLA antigen (W6/32; lanes 2, 4, 6, 8, 11, and 14). A rabbit antiserum against the E3/19K protein was also used (lanes 9, 12, and 15). The migration positions for H-2K^d and HLA antigens, E3/19K protein, and β_2-microglobulin (β_2m) are indicated to the left in the figure. The thick band above the H-2K^d antigen is precipitated only with antibody 34-1-2s but not with other anti-H-2K^d antibodies. See the text for further explanation.

The H-2K^d Antigen Is Not Processed During Intracellular Transport in the Presence of the E3/19K Protein. We examined whether or not the H-2K^d molecule would show an inhibition in carbohydrate processing similar to that observed for HLA antigens. For this purpose, human 293 and 293.12 cells as well as clones 293K^d and 293.12K^d were pulsed for 20 min with [^{35}S]methionine and chased for 2 hr with an excess of cold methionine. Both HLA and H-2K^d antigens were immunoprecipitated with respective antibodies and analyzed by NaDodSO$_4$/PAGE. The HLA antigens from 293 cells showed a marked increase (corresponding to processing of one carbohydrate group) in molecular weight after the chase period (Fig. 2, compare lanes 1 and 2), whereas the same antigens from 293.12 cells did not (compare lanes 3 and 4). A considerable increase in molecular weight (three carbohydrate groups) was seen for the H-2K^d antigen from two 293K^d cell clones (compare lanes 5 and 6 and lanes 7 and 8). In contrast, two 293.12K^d clones showed no increase in the molecular weight of the H-2K^d antigen after the chase period (compare lanes 9 and 10 and lanes 11 and 12). Endoglycosidase H treatment of immunoprecipitated material from 293.12K^d cells confirmed that the H-2K^d antigens do not contain complex type sugars (data not shown). The E3/19K protein was processed to a slightly lower molecular weight after the chase period, indicating cleavage of the core carbohydrates (lanes 3 and 4, 9 and 10, and 11 and 12). Thus, the association of the E3/19K protein with the H-2K^d antigen inhibits the terminal glycosylation of the latter molecules in a similar manner as for HLA antigens (9).

Specificity of Two CTL Clones. For an analysis of how the T-cell response is affected by the H-2K^d–E3/19K association, we used two different CTL clones (10). Clone 10 recognizes HLA-A3 and HLA-A24, whereas clone 12 recognizes HLA-Cw3 as well. Thus, they are not subclones of the same origin but are independent clones. Furthermore, recognition by these CTL clones has been shown to beH-

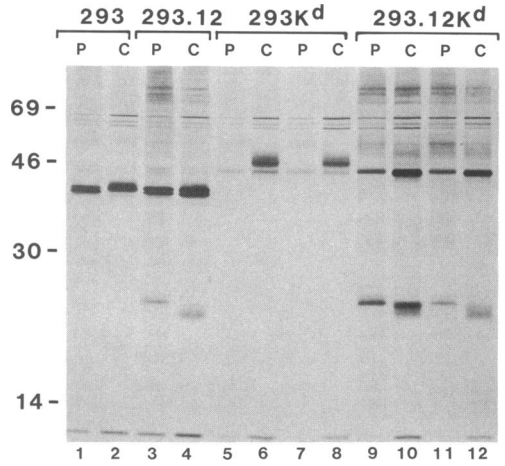

FIG. 2. Inhibition of terminal glycosylation by the E3/19K protein of Ad2. Cells from the lines and clones indicated in the figure were pulse-labeled for 20 min with [^{35}S]methionine and either lysed directly (lanes P) or chased with an excess of cold methionine for an additional 2 hr (lanes C). Antigens were immunoprecipitated with monoclonal antibodies against H-2K^d (20-8-4s; lanes 5–12) and HLA (W6/32; lanes 1–4) antigens and were analyzed by a NaDodSO$_4$/10–15% PAGE. Two individual cell clones were used for 293K^d (lanes 5–8) and 293.12K^d cells (lanes 9–12). The migration pattern of standard molecular weight ($\times 10^{-3}$) markers is shown to the left of the lanes.

2K^d-restricted (10). The mouse mastocytoma cell line P815 (HTR), previously transfected with the *HLA-A2* gene (26, 27) was used as a control because 293 cells express the HLA-A2 antigen.

The four target cell lines, P815TK, P815A2, 293, and 293.12, were ^{51}Cr-labeled and analyzed for their susceptibility to lysis by the two CTL clones (Fig. 3). The P815TK cells expressed a high amount of the endogenous H-2K^d antigens (mean value, 288) but showed background values when tested with an antibody against HLA antigens. Neither CTL clone recognized these cells (Fig. 3*A*). In contrast, the P815A2 cells, which expressed both HLA-A2 (mean value, 120) and H-2K^d (mean value, 246) antigens, were readily lysed by both CTL clones (Fig. 3*B*). The 293 cells, which expressed HLA-A2 antigens (and one additional unknown HLA phenotype) but not H-2K^d antigens, were not lysed (Fig. 3*C*), nor were the 293.12 cells (Fig. 3*D*). Thus, we confirm that both murine CTL clones are HLA specific. In addition, neither of them lyse human 293 cells and 293.12 cells.

Presence of the E3/19K Protein in Cells Correlates with a Lowered Susceptibility to Lysis by CTL Clones. We used CTL clones 10 and 12 described above to investigate whether or not the level of lysis correlates with a reduced amount of HLA/H-2K^d antigens on the cell surface because of association with the E3/19K protein. The amounts of HLA and H-2K^d antigens were measured for several 293K^d and 293.12K^d clones by FACS analysis. The same clones were then examined for their susceptibility to lysis by CTL clones 10 and 12.

The 293K^d target cell clones, 293K^{d}2 and 293K^{d}5, were efficiently lysed by both CTL clones. The 293K^{d}4 cells were also lysed, but not as efficiently. This clone expressed considerably less H-2K^d antigen but more HLA antigens than

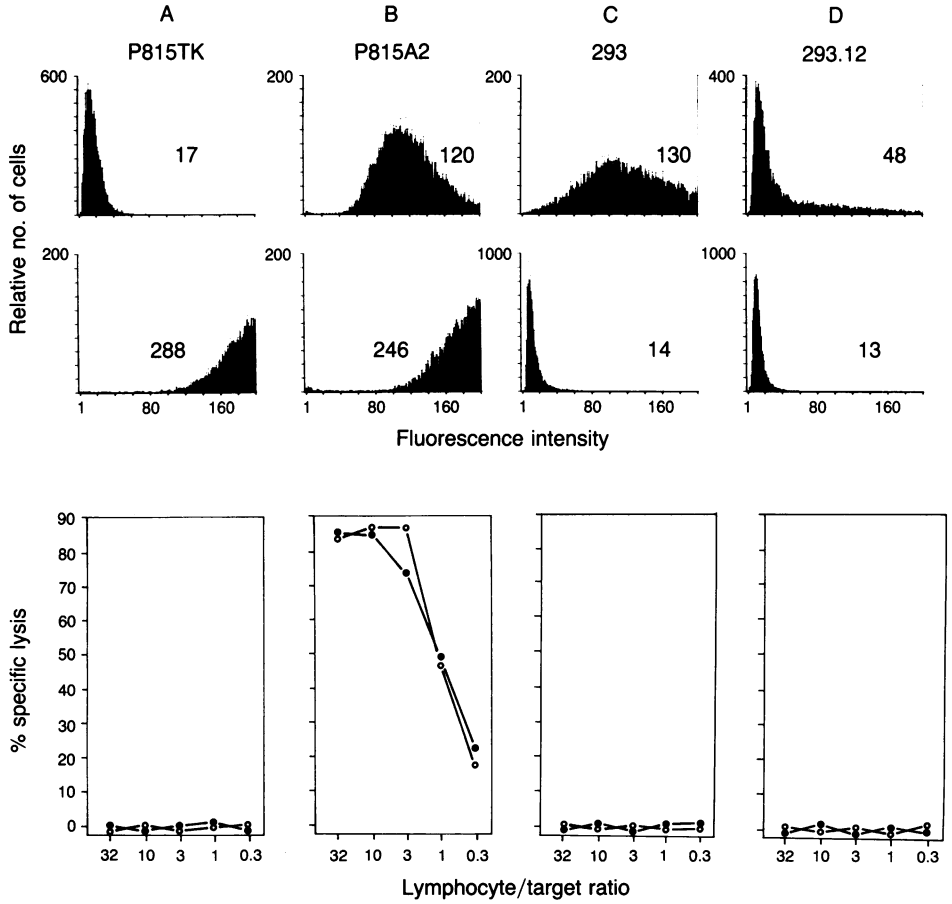

FIG. 3. Specificity of two murine CTL clones. (*Upper*) Cell-surface density of HLA (upper row) and H-2K^d (lower row) antigens as measured by cytofluorography with monoclonal antibodies B9.12 (upper row) and 20-8-4s (lower row). The type of cells used in *A*, *B*, *C*, and *D* are indicated at the top. The number in each graph denotes the mean fluorescence value obtained. (*Lower*) Percentage of specific lysis induced by CTL clone 10 (●) and CTL clone 12 (○).

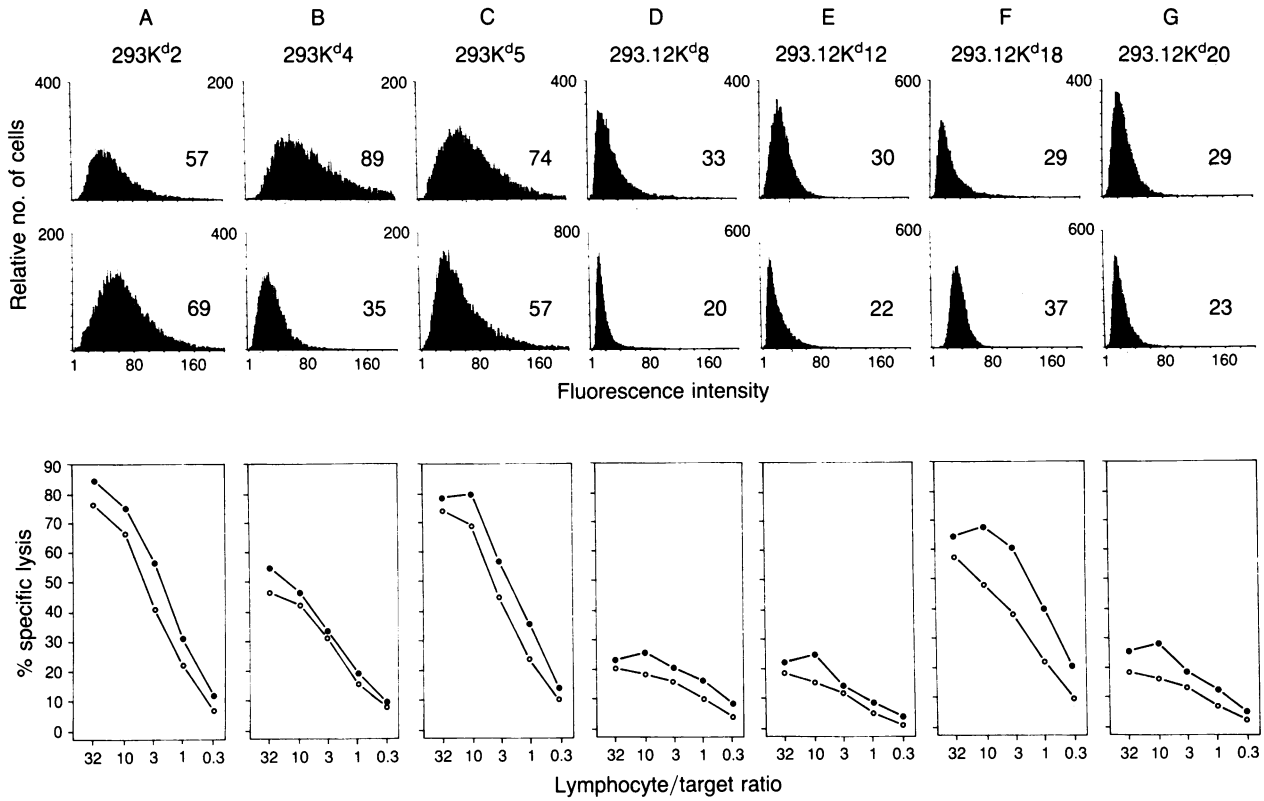

FIG. 4. Reduced cell-surface expression of H-2K^d and HLA antigens correlates with a lowered cytolytic activity. (*Upper*) Several cell clones of both 293K^d and 293.12K^d transfectants were analyzed for their cell-surface density of HLA (upper row) and H-2K^d (lower row) antigens by cytofluorography. The antibodies were the same as in Fig. 3. The numbers in the graphs indicate the mean fluorescence value. (*Lower*) Specific lysis for the individual clones as tested by CTL clone 10 (●) and CTL clone 12 (○).

did the other two clones (Fig. 4, compare *A* and *C* with *B*). Thus, apparently it is the amount of the restriction element, in this case H-2K^d, that is limiting for lysis by these CTL. A marked reduction in lysis of 293.12K^d clones was observed. These cells also expressed the E3/19K protein. This correlates with a lowered cell-surface expression of both HLA antigens and the restricting element H-2K^d (Fig. 4 *D*, *E*, and *G*). In contrast, cells from the target clone 293.12K^{d}18 were lysed efficiently despite the presence of the viral E3/19K protein (Fig. 4*F*). We confirmed that H-2K^d–E3/19K association does occur in this cell line (Fig. 1, lane 13). However, the cell-surface expression of the H-2K^d antigen remained relatively high (mean value, 37) and was comparable to the amount seen in the 293K^{d}4 clone (Fig. 4*B*). This again indicates that the amount of the restricting element H-2K^d is important. We examined a total of seven clones, of which six showed a considerably reduced level of MHC class I antigen on the cell surface and a concomitantly lowered ability to act as targets for CTL lysis. Therefore, we believe that clone 293.12K^{d}18 is exceptional.

DISCUSSION

We have previously demonstrated that terminal glycosylation and cell-surface expression of HLA antigens are inhibited in human cells that express the E3/19K protein of Ad2 (9). This is true for all human cell lines infected with Ad2 so far examined. More recently, others have confirmed our data of inhibition of terminal glycosylation and cell-surface expression of HLA antigens in Ad2-infected HeLa cells (28). These investigators have previously shown that the E3/19K protein is expressed on the cell surface (8). However, we and others have demonstrated that this protein is primarily an intracellular membrane protein (perhaps rough endoplasmic reticulum) in human cells (9, 29, 30). In contrast to the situation in human cells, we were unable to demonstrate an intracellular complex formation between the E3/19K protein and H-2 antigens in two mouse cell lines (L cells and 1T22-6 cells). These cells transfected with the E3/19K gene express normal levels of H-2 class I antigens on their cell surface (H.-G.B. and S.K., unpublished observations). In this study we show that the H-2K^d antigen in human cells does associate with the E3/19K protein (Fig. 1).

The aim of this study was to investigate whether or not the reduced cell-surface densities of class I H-2 and HLA antigens, because of their association with the E3/19K protein of Ad2, would affect the ability of CTL to recognize and kill these cells. For these experiments, we used two murine CTL clones that are both specific for HLA antigens ("foreign" antigen) and H-2K^d-restricted.

We have introduced the *H-2K^d* allele into the human 293 and 293.12 cells. The latter cell line differs from the former in that it expresses the gene for E3/19K (9, 11). Expression of the H-2K^d antigen in amounts comparable to endogenous HLA antigens was verified by immunoprecipitation with monoclonal antibodies. In pulse–chase experiments, we could show that no intracellular processing (terminal glycosylation) of the H-2K^d antigen occurs in the presence of the E3/19K protein (Fig. 2). Moreover, the cell-surface expression of the H-2K^d antigen was considerably reduced in cells expressing the viral protein (Fig. 4).

We have examined to what extent the lowered cell-surface densities of HLA and H-2K^d antigens influence target-cell recognition by two murine CTL clones (Fig. 4). From these experiments we make the following conclusions: (*i*) the inhibition of cell-surface expression of HLA/H-2 class I antigens caused by the E3/19K protein inhibits drastically the ability of the CTL clones to recognize and lyse these target cells; (*ii*) in cells expressing the E3/19K protein, the level of the restricting element, in this case the H-2K^d antigen, is apparently limiting (Fig. 4, compare *B* and *F*); and (*iii*) human

cells expressing mouse class I H-2 antigens can be lysed by mouse CTL.

It is known that Ad1, Ad2, Ad5, and Ad6 can cause latent or persistent infections in humans (31, 32). Thus, the infected cells with their lower level of cell-surface HLA antigens might permit latency of the infection because of a lack of a strong cellular immune response. In this paper we have shown that, at least *in vitro*, the molecular mechanism for lack of T-cell recognition is apparently the complex formation between the E3/19K protein and HLA/H-2 class I antigens. It will be of utmost importance to determine the detailed structure of the E3/19K protein and H-2/HLA antigens that are involved in the binding. With this information, other viral proteins (perhaps from the Herpes virus group) with similar properties can be searched for.

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