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Participation of histocompatibility antigens in capping of molecularly independent cell surface components by their specific antibodies

(immunofluorescence/cell surface mutants/immune cell–cell interactions/histocompatibility restrictions)

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ABSTRACT **The antibody-induced capping of several cell surface components has been investigated by immunofluorescence methods using two mouse cell lines, a parental C58 thymoma line and a mutant derived from it lacking TL and H-2 antigens. Other cell surface components were present in approximately equal amounts on the two cells. Parental cells treated with rabbit antibodies to T200, a major surface glycoprotein, rapidly formed caps containing T200, but the mutant cells similarly treated showed a uniform surface distribution of T200. On the other hand, with a secondary antibody treatment, the T200 on both cells capped equally well. When the indirect T200 caps were examined using a second immunofluorescent stain for H-2, TL, or Thy-1 antigens, it was found that on parental cells all three of these antigens were co-capped with T200; on mutant cells no staining was found for H-2 or TL, as expected, and essentially uniform distribution of Thy-1 was observed. The co-capping of H-2, TL, and Thy-1 antigens with T200 on the parent cell is remarkable, because the first three components are known to be molecularly independent in lymphocyte cell surfaces. The indirect capping of the viral glycoprotein gp 69/71 similarly induced a co-capping of H-2 and TL antigens on the parent cell. These results demonstrate that H-2 and related molecules may co-cap with a variety of independent cell surface antigens. Such co-capping of histocompatibility components could play an important role in a proposed dual recognition mechanism for cell-mediated cytotoxicity reactions and other immunologically important cell–cell interactions.**

When appropriately stimulated, an organism can develop cell-mediated immunity to viral or other antigens. In this process cytotoxic thymus-derived (T) lymphocytes (killer cells) proliferate and, upon contact with target cells bearing the specific antigen, cause the lysis of these cells. A remarkable feature of this immune reaction is that both the stimulator and target cells not only must carry the same antigen (A) but also must possess one or more of the major histocompatibility antigens in common for the cytotoxic reactions to be effective. In the mouse, this major histocompatibility antigen is called H-2. Two hypotheses concerning the mechanism of this and related phenomena have been advanced (1, 2). One hypothesis ("altered-self") is that the killer T cell in the mouse possesses on its surface a single type of recognition molecule, which recognizes a hybrid structure formed between the A molecule and the H-2 molecule on the stimulator and target cells. The second hypothesis ("dual recognition") assumes that the killer T cell carries two different types of recognition molecules on its surface, one, r(A), directed agains the specific antigen A, and the other, r (H-2), against the H-2 molecule on the target cell. Which of these hypotheses is correct is not known.

We have considered a particular version of the dual recognition mechanism which assumes that at some stage the two sets of recognition molecules function simultaneously, and which takes cognizance of the fluid mosaic characteristics of the surface membranes in these cell–cell interactions. This version includes the following elements: (*i*) It is suggested that quite generally a specific cell–cell interaction, mediated by the specific binding of molecules *a* in the one cell surface to molecules *b* in the other, involves the *mutual capping* of the *a* and *b* molecules in their respective membranes into the contact regions between the two cells (3, 4). That is, after some small number of *a–b* bonds form where the two cells make initial contact, lateral diffusion in their respective fluid membranes brings other *a* and *b* molecules into the region of cell contact, where they form additional *a–b* bonds. This results in a collection, or capping, of the *a* and *b* molecules in the contact region. In the special case of immune cytolysis, it is proposed that the r(A) molecules in the membrane of the killer cell bind to and cap the A molecules in the membrane of the target cell, and vice versa, into the regions of cell–cell contact. (*ii*) Within the target cell membrane, the capping of the A molecules induces a "co-capping" of the initially independent molecules of one or more H-2 antigens into the same regions of the membrane (see *Discussion* for possible mechanisms for such co-capping). H-2-like molecules, it is proposed, are unusual in their propensity to be thus co-capped with a variety of A molecules. (*iii*) This capping of H-2 molecules in the target cell membrane induces its specific r(H-2) molecules to bind and collect (cap) in the membrane of the killer cell into the regions of cell–cell contact.

The result of these capping and co-capping events would be to produce an extended region of cell–cell contact in which are collected r(A) and r(H-2) molecules in the membrane of the killer cell, which are bound to the A and H-2 molecules, respectively, collected in the membrane of the target cell. In other words, the sum of the r(A)–A and r(H-2)–H-2 bonds so formed would define the strength and surface area of the cell–cell contact. In the absence of the r(A)–A interaction and consequent capping events, the r(H-2)–H-2 interaction itself, for one of several possible reasons, might not lead to either a sufficiently stable or a functionally suitable link between the two cells. However, by the proposed mechanism, the dual recognition system would produce a longer-lived and more intensive interaction between the two cells, which could be critical for the expression of cytotoxicity. In this way, the dual recognition system would be mechanistically advantageous.

Among the predictions of this scheme, relevant to element *ii* above, is that the H-2 molecules in mouse lymphocyte membranes may co-cap with certain independent membrane

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receptors when the latter are capped by their specific soluble antibodies. If this were true, it would be in conflict with the currently accepted view that molecularly independent receptors cap independently of one another (cf. ref. 5). In our studies to investigate this prediction, it has been useful to compare the capping properties of two closely related C58 mouse thymoma cell lines, R1(TL^+) and its mutant R1(TL^-). The mutant (6) is of interest because it is deficient in cell surface TL and H-2 antigens, while on the other hand, several other antigens are present in essentially the same amounts in the surfaces of the two cell types (6, 7). One of these antigens, T200 (8, 9), a major glycoprotein in these thymoma cells and in normal T lymphocytes, was a major focus of interest in the present studies. The preliminary results we have obtained support the thesis that H-2 molecules, among others, may indeed co-cap with independent antigen molecules in cell membranes, and therefore they lend some plausibility to the version of the dual recognition mechanism in immune cytolysis that we have proposed.

MATERIALS AND METHODS

Cell Lines. R1(TL^+) is a spontaneous C58 thymoma that has been adapted to tissue culture (10). The R1(TL^-) cell line was derived from R1(TL^+) by complement-mediated immune selection against the TL antigen (6) and lacks detectable cell surface TL and H-2^k antigen as determined both by quantitative cytotoxic absorption (6) and by immunoprecipitation of extracts of cells labeled by lactoperoxidase-catalyzed iodination (7). The R1(TL^-) cell line is insensitive to cell-mediated cytotoxicity directed either against the H-2supnk antigen itself (11, 12) or against minor histocompatibility antigens (11) or the trinitrophenyl determinant (12).

Both R1(TL^+) and R1(TL^-) were grown as suspension cultures in Dulbecco's-modified Eagle's medium with 10% horse serum (13). Other cell lines, used for absorption of antisera, have been described (10, 13, 14).

Antibodies. The mouse anti-H-2^k serum was prepared by injecting (C3H.SW × B10.D2-new) mice repeatedly with the C3H sarcoma BP8. This antiserum recognizes (probably) only both the K and D ends of the H-2^k complex. The serum was absorbed three times with a 1/3 volume of a pool of the H-2^d myeloma S194/2·CMD·3·3·3 and the H-2^b lymphoma EL4·BU·1 to absorb autoantibodies and antiviral antibodies and was specific for H-2^k cell lines in direct cytotoxic tests.

Mouse anti-TL 1, 2, 3 was prepared by repeated injection of C57BL/6 × A-Tla^b) mice with the A strain spontaneous leukemia ASL1. The serum was absorbed three times with 1/3 volume of a pool of the lymphoma cell lines BW5147·G·1·4 and EL4·BU·1 to remove antibodies against murine leukemia virus and auto-antibodies. The absorbed serum was specific for TL^+ cell lines in direct cytotoxic tests.

Mouse anti-Thy-1.2 antiserum was prepared by intraperitoneal immunization of AKR/J mice with C3H/HeJ normal thymocytes. The serum was absorbed three times with 1/3 its volume of the Thy-1.1 lymphoma BW5147·G·1·4 to remove contaminating autoantibodies and antibodies against murine leukemia virus. No immunofluorescent staining was seen with Thy-1.2-negative mutant cell lines (14) after absorption.

Goat antiserum against Rauscher leukemia virus glycoprotein gp 69/71 (lot no. 6S-385) was obtained from the Office of Program Resources and Logistics, National Cancer Institute.

Rabbit antiserum against T200 glycoprotein isolated from the Thy-1.1, TL^- lymphoma BW5147·G·1·4 was prepared by immunization with the partially purified glycoprotein obtained by fractionation of sodium deoxycholate-solubilized membrane glycoproteins of the cells on Sepharose 6B. Before use the anti-T200 serum was absorbed with S194/2·CMD·3·3·3 myeloma cells (15). Both anti-gp 69/71 and anti-T200 antisera precipitated a single labeled species from detergent extracts of BW5147 cells surface-labeled by lactoperoxidase-catalyzed iodination (Fig. 1). There is no evidence that either serum contains antibodies directed against the other cell surface antigens used in these studies. In some experiments, anti-T200 serum was absorbed exhaustively with AKR mouse liver homogenate to eliminate any possibility that the serum contained trace amounts of antibodies against H-2, and this treatment did not modify the results obtained.

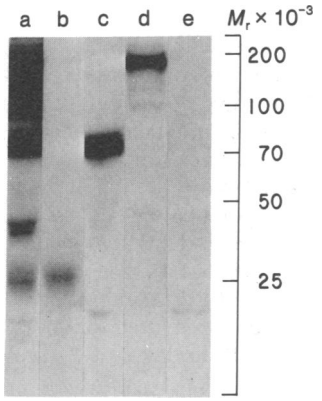

FIG. 1. Specificity of antisera against Thy-1, gp 69/71, and T200 glycoprotein. Immunoprecipitates were prepared with various antisera from a detergent extract of BW5147 cells labeled by lactoperoxidase-catalyzed iodination and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (9). Shown are autoradiographs of the labeled species precipitated by (gel a) an antiserum against whole BW5147 cells, (gel b) anti-Thy-1 glycoprotein serum (15), (gel c) anti-gp 69/71 serum, (gel d) anti-T200 serum, (gel e) normal rabbit serum. Only one major band is specifically precipitated in gels b, c, and d.

Fluorescent Labeling Reagents. Affinity-purified goat antibodies to rabbit IgG (for the T200 experiments) and rabbit antibodies to goat IgG (for the gp 69/71 experiments) were conjugated to lissamine rhodamine B sulfonyl chloride according to published procedures (16). For indirect staining in the co-capping experiments, goat antibodies raised to mouse IgG were affinity purified and conjugated with fluorescein isothiocyanate.

Capping Experiments. For the direct capping of the T200 antigens, cells were treated with an appropriate dilution of rabbit anti-T200 antiserum at 0° for 30 min. The cells were then washed three times at 0° with phosphate-buffered saline containing 0.2% bovine serum albumin to remove unbound antiserum components, after which they were incubated at 37° for 5 min, fixed for 15 min in 1.5% (wt/vol) paraformaldehyde, and washed three times with phosphate-buffered saline containing 0.02 M glycine to quench any remaining aldehyde groups. Rhodamine-conjugated goat antibodies to rabbit IgG were then added to the cells and incubated for 30 min before washing and examination. For indirect capping of T200 or gp 69/71 or aminopeptidase, cells were first treated with rabbit antisera to T200, or goat antisera to gp 69/71, or goat antisera to aminopeptidase, respectively, at 0° for 30 min, and washed at 0° as described above. The cells were then incubated for another 30 min at 0° with rhodamine-conjugated goat antibodies to rabbit IgG in the case of T200, or with rhodamine-conjugated rabbit antibodies to goat IgG in the case of gp 69/71 or aminopeptidase. After washing at 0°, the cells were incubated at 37° for 5 min. Subsequent to these treatments, the cells were fixed with formaldehyde.

For the co-capping experiments, cells that had been directly and indirectly capped for T200, or indirectly capped for gp

69/71 or aminopeptidase and then fixed as described above, were washed three times with phosphate-buffered saline containing 0.02 M glycine, and were then treated with mouse antisera to $H2^k$, TL, or Thy-1 at room temperature for 30 min. After washing, the cells were incubated with fluorescein-conjugated goat antibodies to mouse IgG.

Fluorescence Microscopy. The cells that had been labeled in this manner were examined with a Zeiss Photoscope III using a 63× oil immersion lens and epi-illumination. Fluorescein and rhodamine fluorescences were excited with an Osram HBO 50-W bulb, and the filter combinations CZ487710 and CZ487714, respectively, were used for observation. Photography was performed with Kodak plus-X film.

RESULTS

When rabbit antibodies to the mouse T200 antigen were bound to the surfaces of the parental thymoma cells, they induced the rapid direct capping of that antigen at 37° (Fig. 2*A*). This was revealed by fixing the treated cells, and staining them with the fluorescein-conjugated goat antibodies to rabbit IgG. On the other hand, the same treatment of the mutant cells resulted in the binding of the rabbit antibodies but no capping (Fig. 2*B*), even after 1 hr of observation. When, however, the fluorescein-conjugated goat antibodies were added to unfixed cells that had first been treated with the rabbit antibodies to T200, and the cells were then incubated at 37° before fixation, *both* the parental and mutant cells showed essentially equivalent indirect capping of the T200 antigen (Fig. 2 *C* and *D*). The direct and indirect caps on the parental cells were somewhat different in appearance, with the latter exhibiting a more patchy structure.

For co-capping experiments, fixed cells such as those in Fig. 2 *C* and *D*, containing indirect T200 caps induced by secondary antibody treatment (this time with rhodamine-conjugated goat antibodies to rabbit Ig), were then treated with mouse alloantibodies directed to either the H-2, TL, or Thy-1 antigens present on the parental cells, and then with fluorescein-conjugated goat antibodies to mouse Ig. The H-2 (Fig. 2 *E* and *F*), the TL (Fig. 2 *G* and *H*) and the Thy-1 (Fig. 2 *I* and *J*) fluorescein staining patterns were uniformly found to be co-extensive with the T200 rhodamine caps on the parental cells. If normal mouse IgG was substituted for the mouse alloantibodies, no fluorescein staining was observed. The same treatments of the mutant cells showed no staining for H-2 (Fig. 2 *K* and *L*) or for TL (not shown), as expected from the absence of these antigens from the mutant cell surface, and a variable but largely uniform surface staining for Thy-1 (Fig. 2 *M* and *N*). In the last case, although in different cells different degrees of redistribution of the Thy-1 antigen into the region of the T200 caps was observed, in many cells the majority of the Thy-1 remained uniformly distributed, a very different result from that obtained with the parental cells (Fig. 2*J*).

The capping of the gp 69/71 antigen required a secondary antibody on both the parental (Fig. 3*A*) and the mutant (Fig. 3*C*) cells. When such capped and fixed cells were then stained for H-2, it was found to be co-capped with the gp 69/71 on the parental cells (Fig. 3 *A* and *B*), and was absent, as expected, from the mutant cell (Fig. 3*D*). Similar results were obtained (not shown) for the TL antigen as for the H-2.

DISCUSSION

The two C58 thymoma cell lines used in this study have been characterized in detail elsewhere (6, 7, 11, 12). Briefly, the mutant cell line R1(TL^-) had undetectable amounts (<3% of the parental line) of either the TL or H-2 antigens, by radiolabeling and serological techniques. On the other hand, the mutant cells had approximately the parental amounts of Thy-1.2 (6), gp 69/71 (R. Hyman, unpublished data), and T200 antigens (7) expressed on their surfaces. These features were confirmed qualitatively by the immunofluorescence studies reported in this paper.

Despite the similarity in amounts of the T200 antigen on the surfaces of the parent and mutant cells, a remarkable difference in the capping behavior of that antigen was observed. T200 could be directly capped by its rabbit antibodies on the parent but not on the mutant cells (Fig. 2 *A* and *B*). That this difference was not due to a generalized defect in the capping capacity of the mutant cells was shown in at least two ways. First, the *indirect* capping of the T200 antigen, using both primary and secondary antibody reagents, elicited closely similar capping behavior on the parental and mutant cells (Fig. 2 *C* and *D*). Second, the capping by fluorescein-conjugated concanavalin A of both cells was indistinguishable (not shown). The inference is that, if the surface properties of the two cell lines are indeed otherwise indistinguishable, the absence of the H-2 and TL antigens from the mutant cell is directly associated with the inability to cap its T200 antigen with the primary antibody.

Of particular interest is the finding that the H-2 (Fig. 2 *E* and *F*), TL (Fig. 2 *G* and *H*), and Thy-1 (Fig. 2 *I* and *J*) antigens co-capped with the T200 antigen when the latter was capped either directly (not shown) or indirectly by specific antibodies applied to the parental cell. That these co-capping effects reflect a true collection of H-2, TL, and Thy-1 molecules with the T200 molecules into the caps is indicated by the results obtained when the mutant cells were similarly treated. The absence of staining for H-2 (Fig. 2 *K* and *L*) and for TL (not shown) in the indirect T200 caps, while expected for the $H\text{-}2^-$, TL^- mutant, shows that the H-2 and TL staining on the parental cell caps cannot be attributed to such artifacts as (*i*) nonspecific characteristics of the anti-H-2 or anti-TL antisera, or (*ii*) co-capping of Fc receptors with the T200 antigen. Furthermore, the different behavior of the Thy-1 antigen (present to a comparable, if not equal, extent on both kinds of cells) in *not* extensively co-capping with T200 on the mutant cell (Fig. 2 *M* and *N*) imparts added significance to its extensive co-capping with T200 on the parental cell (Fig. 2 *I* and *J*).

What are the molecular events involved in these co-capping phenomena? The current view of capping (5) is that components that are molecularly independent of one another in the unperturbed cell membrane are independently capped. That is, upon capping a particular membrane component by its specific antibodies, only those other membrane components are expected to be co-capped with the first that form specific molecular complexes with it in the membrane. Thus, co-capping has in the past been taken as strong, if not conclusive, evidence for a direct molecular interaction between the components that co-cap. If so interpreted, however, our result would imply that at least four antigenic components: T200, H-2, TL, and Thy-1, in the case of the parental cell, formed a molecular complex in the membrane. On the contrary, it has already been shown by their independent capping and independent reexpression after surface modulation that TL, H-2, and Thy-1 antigens are molecularly independent components in thymus cell membranes (17). Our observations indicate that the co-capping of certain molecularly independent components may indeed occur under appropriate circumstances.

The mechanisms of these unusual co-capping phenomena are at present obscure. A simple physical sweeping of other components in the membrane into the cap formed by the T200 antigen does not alone account for all of the observed results,

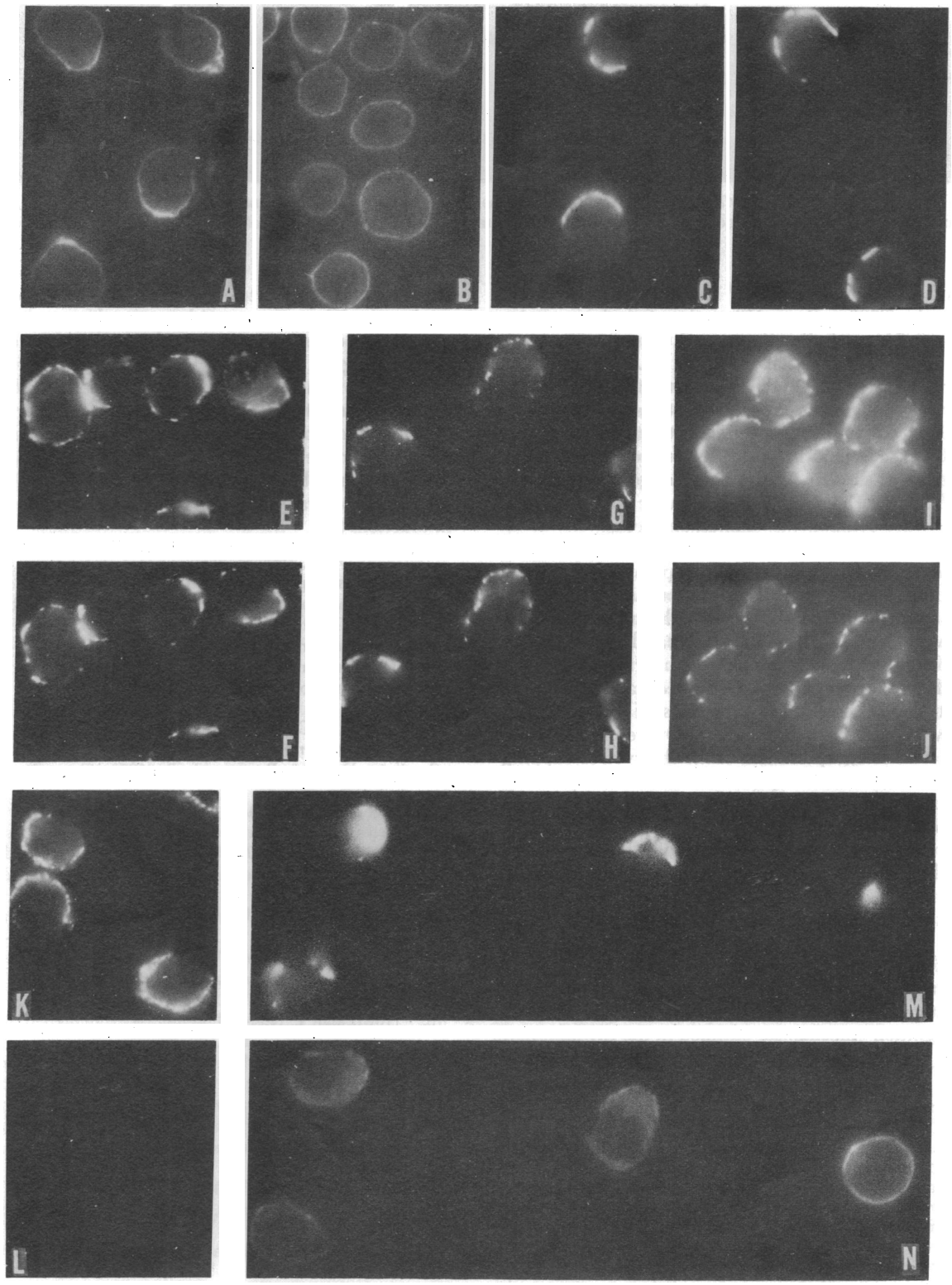

FIG. 2. Fluorescence microscopy. (×800.)(*A*) Direct capping by anti-T200 antibodies on parental cells. (*B*) Absence of direct capping by anti-T200 antibodies on mutant cells. (*C*) Indirect capping of the T200 antigen on parental cells. (*D*) Indirect capping of the T200 antigen on mutant cells. (*E*) Indirect capping of the T200 antigen on parental cells; (*F*) on the same cells, co-capping of the H-2 antigen. (*G*) Indirect capping of the T200 antigen on parental cells; (*H*) on the same cells, co-capping of the TL antigen. (*I*) Indirect capping of the T200 antigen on parental cells; (*J*) on the same cells, co-capping of the Thy-1 antigen. (*K*) Indirect capping of the T200 antigen on the mutant cells; (*L*) the absence of staining for the H-2 antigen on the same cells. (*M*) Indirect capping of the T200 antigen on the mutant cells; (*N*) the general absence of co-capping of the Thy-1 antigen on the same cells. Although there appears to be some degree of concentration of the Thy-1 staining associated with the T200 caps in some of the cells, there is a clear difference from the extensive co-capping observed in *J*.

particularly for the fact that in the absence of H-2 and TL molecules the T200 molecules cannot be capped directly. Some kind of complex interaction, perhaps between small clusters of T200 molecules and other membrane components, may be

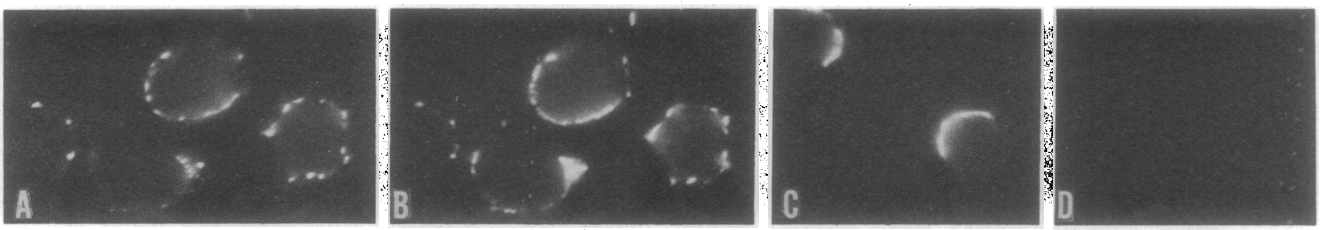

FIG. 3. Fluorescence microscopy. (×780.)(*A*) Indirect capping of the gp 69/71 antigen on the parental cells; (*B*) on the same cells, co-capping of the H-2 antigen. (*C*) Indirect capping of the gp 69/71 antigen on the mutant cells; (*D*) the absence of staining for the H-2 antigen on the same cells.

involved in the co-capping observed.¶ If so, this is not a property confined to the T200 antigen. When the viral glycoprotein antigen gp 69/71 was indirectly capped using specific goat antibodies on the parental cells, H-2 and TL antigens were co-capped with it (Fig. 3). On the other hand, it is particularly interesting that with the enzyme aminopeptidase as the surface antigen (19), its capping with specific goat antibodies left the H-2 and TL antigens uniformly dispersed on the surfaces of the parental cells (not shown). The factors that govern these co-capping phenomena clearly need systematic study, using a variety of surface components, ligands, and cells.

Despite present uncertainties about the detailed mechanisms involved, however, our findings on co-capping carry several important implications. From the observation that viral antigens apparently co-cap with the H-2 antigen on the surfaces of tumor cells, Schrader *et al.* (20) have inferred that the two types of molecules form a molecular complex (a "hybrid antigen") on the cell surface. These experiments have been taken, therefore, to support the "altered-self" hypothesis of cell-mediated cytotoxicity reactions (see *Introduction*). Our results, however, indicate that other interpretations of the observed co-capping are possible, and that such experiments therefore do not necessarily support the "altered-self" hypothesis. In another connection, the sporadic inclusion of H-2 antigenic determinants on C type virus particles (21), which form by a process of assembly and budding from the cell membrane, may likewise be the result of a nonstoichiometric co-capping of H-2 with the viral capsid proteins during the viral assembly process.‖

Finally, our findings that H-2 and TL not only can co-cap with independent surface antigens, but also, as in the case of the T200 antigen, can affect the capping efficiency of another surface antigen, lend some plausibility to the version of the "dual recognition" mechanism outlined in the *Introduction*. This is not the place to elaborate upon or to defend that mechanism. The point we wish to stress here is that H-2 and related molecules may co-cap with a variety of independent surface antigens, and that such co-capping could play an important role in a dual-recognition scheme of cell-mediated cytotoxicity reactions. Other products of the major histocompatibility gene complex may also exhibit co-capping with appropriate surface components, and in a similar manner, such co-capping may be essential to the involvement of these gene products in several other immunologically important cell–cell interactions (2).

¶ A possible explanation of this different capping behavior may be given in terms of a recently developed theory of the capping phenomenon (18). In this theory, the clustering of a membrane receptor into a small patch causes it to become linked across the membrane to actin and myosin components on the inner membrane surface. These actin- and myosin-linked patches are then collected into a cap by activation of the actin–myosin sliding filament mechanism. When the primary antibody to the T200 antigen is added to the parental cell, the clustering of the T200 antigen together with H-2, TL, and other membrane molecules may produce a sufficiently large or "sticky" patch to allow the transmembrane linkage to actin-myosin to form; whereas with the mutant cell, the clustering of the T200 antigen in the absence of H-2 and TL may produce a smaller or less "sticky" patch. In the latter case, however, the addition of the secondary antibody could produce larger patches that would be able to form the transmembrane linkages.

‖ It has been observed that a selective incorporation of H-2D compared to H-2K antigens occurs in these virus particles (21). It is possible that the molecularly independent H-2D and H-2K antigens (22), despite their close similarity, do not co-cap to an equal extent in our experiments when T200 and other surface antigens are capped on the parental cells; specific anti-H2K and anti-H2D antibodies would be required to investigate this point.

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