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A Fluorescence Study on the Mobility of Surface Antigens of Untreated Tumor Cells and of Tumor Cells Undergoing Cell-Mediated Lysis

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Summary. Heterologous (rabbit) antibodies were raised against murine P-815 mastocytoma cells of DBA/2 origin. Antisera and IgG preparations were highly cytotoxic, whereas Fab fragments thereof lost all activity. Fab fragments also showed a much lower avidity than IgG, both for tumor and normal DBA/2 and C57 spleen cells as measured by the release of iodinated Fab and IgG. Both preparations bound specifically to P-815 cells since they were capable of inhibiting T cell-mediated target cell lysis. The binding of IgG and monovalent Fab fragments was studied by fluorescence. Rhodamine-coupled IgG bound homogeneously in the cold and quickly formed patches upon warming but did not form caps even after prolonged incubation at 37° C.

Rhodamine-coupled Fab fragments also bound homogeneously. Their distribution was unaltered after incubation at 37° C even when tumor cells formed uropod-like tails. Fab fragments, however, could be induced to cap with a second and third antibody layer.

P-815 cells labeled with rhodamine-coupled Fab fragments were incubated with cytolytic T cells (CTL). The conjugates formed between CTL and fluorescent target cells were observed. No gross redistribution of surface antigens on target cells was observed even at late stages of the lytic process.

CTL, therefore, do not seem to operate via a redistribution of surface antigens.

Key words: Tumor antigens – Cell cytotoxicity – T cells – Fluorescence – Membrane fluidity

Introduction

The distribution and mobility of surface antigens of tumor cells may have important consequences for processes such as growth, metastasis and escape

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from host immune mechanisms (Nicolson 1976). Murine ascites tumors such as the P-815 mastocytoma have been shown to be equipped at their surface not only with a set of H-2 antigens but also to possess tumor-associated transplantation antigens (TATA) (Al-Rammahy and Levy 1979, Clemetson et al. 1976). The mobility of such surface antigens can be studied conveniently by the process of ligand-induced capping (Taylor et al. 1971). Essentially 2 types of capping can be distinguished (Schreiner and Unanue 1977): one type clearly dependent on the contractile apparatus of the cell, occurring for example in rapid capping of surface Ig and Fc-receptors, another represented by H-2 capping, which is much slower, usually requiring 2 layers of crosslinking ligands. This latter form seems to be related to some form of lipid flow within the membrane perhaps secondary to motility. Its linkage to the contractile apparatus of the cell is still disputed (Braun et al. 1978; Koch and Smith 1978). It was the first aim of this study to clearly document the requirements to achieve H-2 patching and capping on tumor cells by the use of mono- and bivalent fluorescent reagents.

The second aim of this study was to reexamine the hypothesis that H-2 capping was instrumental in the process of target cell lysis mediated by cytotoxic T cells (CTL) (Berke and Fishelson 1975). Indeed, as reviewed by Berke (1979), Cerottini and Brunner (1977), Golstein and Smith (1977), Henney (1977) and Martz (1977), CTL recognize and bind specifically to H-2 antigens present at target cell surfaces. The firm binding of the CTL to the target cell is followed by the delivery of a lethal hit resulting eventually in target cell lysis. The nature of the lethal hit is not known. Current opinion is divided as to whether the firm attachment by itself would be able to generate a lethal hit or whether this was only a prerequisite for a secondary process to occur. In the former case a redistribution and/or altered mobility of membrane antigens might be expected and could be the cause for the altered permeability properties of the target cell.

Materials and Methods

Animals. C57 Bl/6 (H-2^b) and DBA/2 (H-2^b) mice and New Zealand white rabbits were provided by the Institut für biologisch-medizinische Forschung, Füllinsdorf. Mice were raised under spf conditions but kept conventionally during experimentation.

Tumor. Mastocytoma P-815 of DBA/2 origin was maintained by continuous in vitro culture and passaged twice weekly.

Immunization. Rabbit anti-P-815 antisera were raised by 3 i.v. injections of 10^8 irridiated (6,600 rads) P-815 cells at weekly intervals. Animals were bled one week after the last injection. Normal, untreated rabbits served as controls.

Production and Purification of Monovalent Fab Fragments of Rabbit Anti-P-815 Antibodies. Decomplemented pools of suitable rabbit antisera were precipitated at room temperature three times with $(NH_4)_2SO_4$ at 33% saturation. The precipitate was redissolved in borate saline buffer and dialysed against this buffer for 48 h. The buffer solution was then replaced by 0.01 M phosphate buffer, pH 6.5, and the sample subjected to DE 52 ion exchange chromotagraphy (Whatman Inc., Springfield Mill, Maidstone, Kent, England). Eluates at 0.01, 0.02 and 0.03 M phosphate buffer were collected, concentrated and tested for activity in microcytotoxicity tests and for purity in immunoelectrophore-

sis (see below). Electrophoretically pure preparations were lyophilised and pooled. Digestion with papain (mercuripapain, Worthington Biochemical Corporation, Freehold, N.J.) was carried out by the method of Porter (1959). The digest was dialyzed against water for 48 h for precipitation of most Fc fragments and then against acetate buffer, 0.1 M, pH 5.5. The sample was then applied to a CM 52 (Whatman) column using 11 g of preswollen gel for 100 mg protein. After the elution of the first protein peak at 0.1 M buffer (containing Fab fragments and some undigested IgG) a gradient ranging from 0.1 to 1.0 M acetate buffer was applied which resulted in the elution of 2 further peaks containing Fab fragments (peak 2) and a small amount of Fc fragments (peak 3). Peaks containing Fab fragments were twice chromatographed on a 2.5×100 cm Sephacryl S-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden) running 50 mg protein in 0.4 Tris buffer, pH 8.0, in order to remove undigested Ig. IgG and Fab fragments prepared accordingly from normal rabbit sera served as controls.

Fab fragment preparations that were judged pure in SDS-polyacrylamide gel electrophoresis, devoid of any cytotoxic activity in microcytotoxicity tests and active in fluorescence (binding to P-815 cells developed by fluorescent goat anti-rabbit IgG antibody, see below) were coupled to rhodamine (TRITC isomer R, crystalline, Nordic Immunology, Tilburg, The Netherlands) using the technique of Amante et al. (1972). Unbound reagent was removed after coupling by passage through a 1×50 cm G-50 sephadex column (Pharmacia) and protein peaks collected, concentrated and dialyzed against PBS.

Other Antibodies. Goat anti-rabbit IgG and rabbit anti-goat IgG antibodies (IgG preparations) were purchased from Nordic.

Immunoelectrophoresis and Polyacrylamide Gel Electrophoresis. Immunoelectrophoresis was performed in 1.5% agarose, 0.05 M Na barbital, pH 8.2, and the slides developed with a swine anti-rabbit serum protein antibody (Dakopatts A/S, Copenhagen, Denmark). Polyacrylamide gel electrophoresis in 0.1% sodium sulfate (SDS) was performed according to Laemmli and Favre (1973) using 10% acrylamide in the separating and 3% in the stacking gel in a vertical slab-gel apparatus. Sample buffer was 62.5 mM tris, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol and 15% glycerol. Gels were stained in 0.25% Coomassie Brilliant Blue R 250 (Schwarz/Mann, Orangeburg, New York) and destained in a 7% acetic acid/5% methanol solution.

Iodination Procedures. Fab fragments were labeled with the Bolton-Hunter reagent (IM 861, The Radiochemical Centre, Amersham, England). Between 10 and 100 µl of a benzene/DMF solution were evaporated in a conical glass tube under a stream of N_2 and used for the labeling of 1.5 mg protein, followed by extensive dialysis.

Microcytotoxicity Tests. Complement-dependent cytotoxicity of antibody preparations was determined by the trypan blue dye exclusion test (Boyse et al. 1964) using normal rabbit serum (1/15 final dilution) as source of complement. Prior to use the complement was treated with agarose (Cohen and Schlesinger 1970) and then absorbed extensively with mouse spleen cells until the cytotoxic titer on C57 or DBA/2 thymus cells had fallen below 10% after 45 min incubation at the above concentration.

Methods of Cell Preparation, Ficoll Gradient and Nylon Wool Purification, Cell Culture Methods. Established methods were used as described previously (Matter 1976; Matter 1978). In general, C57 spleen cells of animals which had rejected an inoculum of 10^7 P-815 cells 2–3 months earlier were cocultured with 1,200 rad irradiated DBA/2 spleen cells. Culture medium was RPMI 1640 supplemented with 10% fetal calf serum, 5×10^{-5} 2-mercapto-ethanol, antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml) and 20 mM Hepes buffer (Pharmacia). 4–5 days later cells were harvested, washed, freed from dead cells on Ficoll gradients and subsequently freed from adherent cells on nylon wool columns. Such cells were referred to as cytotoxic T lymphocytes (CTL).

Chromium Release Assay. This assay was performed according to Brunner et al. (1968) and Cerottini and Brunner (1971) with slight modifications which have been described (Matter 1976; Matter 1978).

Incubations for Fluorescence Staining of Cells. Typically, samples of 2×10^6 P-815 were stained in 50 µl culture medium containing between 2 and 10 µl of an antibody solution which contained between 1 and 5 mg protein/ml for 1 h at temperatures indicated in the results. Cells were then washed twice and resuspended for observation or incubated similarly with a second and third antibody.

In order to demonstrate effects of CTL on target antigens of tumor cells, stained and washed P-815 were incubated with CTL at a ratio of 1:5, spun down in a conical tube and incubated at 37° C for varying periods of time as indicated in the results, gently resuspended and immediately observed.

Fluorescence Microscopy. A Leitz vertical fluorescence illuminator (Leitz, Wetzlar, West Germany) equipped with a dichroic beam splitter (λH at 580 nm) was used. Green excitation light was generated with a mercury super pressure lamp HBO 200 W/4 and selected with a 2 mm BG 36 colored glass filter and an interference band filter PAL 544 nm (half width 21 nm, peak transmittance 59%) manufactured by Schott und Gen., Mainz, West Germany. The suppressor filter was a K 590 colored glass filter. All observations were performed using a 40× oil immersion lens.

EM Microscopy and Autoradiography. These techniques were performed as described in detail elsewhere (Matter et al. 1972).

Results

1. Anti-P-815 Xenoantibodies: Range of Specificities

Hyperimmune rabbit antisera to P-815 mastocytoma cells were prepared as described in the Materials and Methods section.

Their relative strength and degree of specificity was examined by complement-mediated lysis of various target cells in microcytotoxicity tests. Figure 1 A shows the typical activity of pools of such sera before and after purification. Both DBA/2 and C57 spleen cells were lysed indiscriminately up to final dilutions of 1/384 whereas lysis of P-815 showed a prozone. Purified IgG preparations showed somewhat less activity than whole antiserum, probably because of a lower concentration since the IgG concentration/ml serum was around 20–25 mg. IgG preparations did not show any prozones, possibly due to elimination of immune complexes. Fab preparations, when purified completely from remaining undigested antibody by successive passages on chromatography gels, did not exhibit any cytotoxic activity.

The range of specificities produced was examined by absorptions and subsequent tests in microcytotoxicity assays. As Fig. 1 B shows, 10× absorption with DBA/2 spleen cells (volume of packed cells to volume of serum = 1:1) were necessary to eliminate activity on DBA/2 cells. These absorptions also abolished all activity towards C57 spleen cells, probably by removing antibodies to one or several common xenoantigens. The residual activity on P-815 cells was suggestive of a tumor-associated transplantation antigen (TATA) (Al-Ramahy and Levy 1979, Clemetson et al. 1976). This activity was, however, completely lost after 6 further absorptions. It should be mentioned, however, that this serum retained a clearly recognizable activity on 5–10% P-815 cells by fluorescence when used in conjunction with a fluorescent goat anti-rabbit Ig antibody (see below, fluorescence results).

A series of 10 absorptions with C57 spleen cells (Fig. 1 C) resulted in the

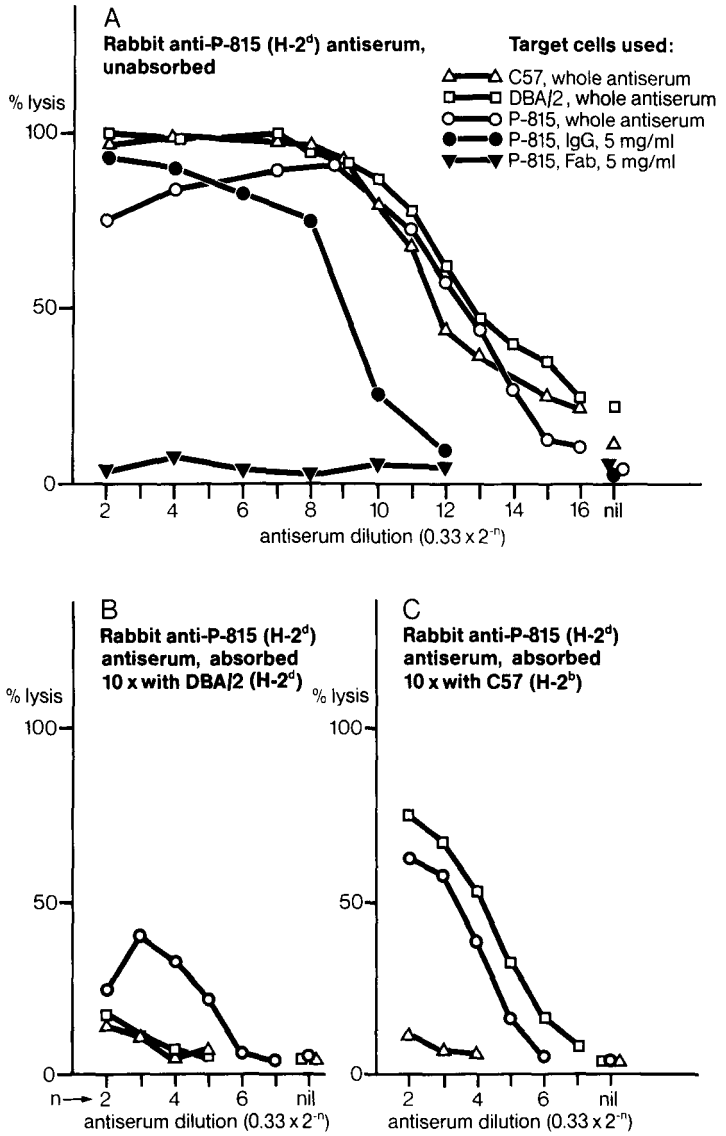


Fig. 1. Complement-dependent microcytotoxicity assays with various target cells and unabsorbed (panel A) or absorbed (panel B, C) antisera raised in rabbits against P-815 cells. Results with IgG and Fab fragments prepared from unabsorbed antisera are also shown on panel A

complete loss of cytotoxic activity towards these latter cells, but left unaffected the considerable activity towards DBA/2 and P-815 cells. It is most likely that antibodies against common xenoantigens and public specificities were removed by these absorptions but that C57 cells were unable to remove the specific antibodies directed towards private alloantigens.

It can be concluded from these studies that rabbits are able to distinguish xenoantigens, alloantigens and TATA on murine tumor cells.

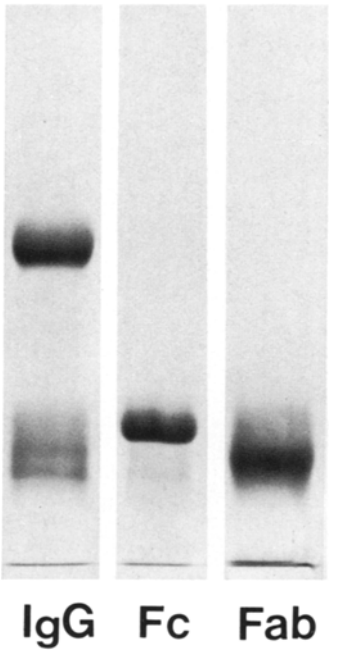


Fig. 2. SDS-polyacrylamide gel electrophoresis. Coomassie blue staining pattern of IgG, Fc and Fab fragments (40 μg per each band) prepared from rabbit anti-P-815 antisera

2. IgG and Fab Preparations of Anti-P-815 Antibodies: Purity, Avidity and Effects on T Cell-Mediated Lysis

Figure 2 shows the chromatogram of Fab and IgG preparations in SDS-polyacrylamide gel electrophoresis. As mentioned above, electrophoretically pure Fab preparations were inactive in complement-mediated cytotoxicity. The following experiments were designed to demonstrate an immunologically specific binding of Fab fragments to P-815 cells:

a) Fab fragments were unable to inhibit the cytotoxic activity of IgG preparations even in the presence of a ten-fold excess (w/w) of Fab (data not shown). This was presumably caused by the relatively low affinity of Fab fragments versus IgG which was examined as follows (Fig. 3): Samples of P-815 cells (2×10^6/tube) were incubated for 2 h with 50 μl of a Tagit-iodinated Fab or IgG preparation containing roughly 50 μg of protein. Between 0.2 and 0.6% of Fab and 0.9–2.0% of IgG radioactivity were retained under such conditions. After 2 washings the release of radioactivity from the cells at 37° C in culture medium was measured by taking samples every 30 min from the supernatant while washing the cells. The obtained percentage of input radioactivity was plotted cumulatively. The viability of the cells during the time of the experiment was unaffected. As can be seen from Fig. 3, iodinated Fab was released to a large degree from the cellular surface within 3 h, while IgG was retained by more than 60% after the same interval.

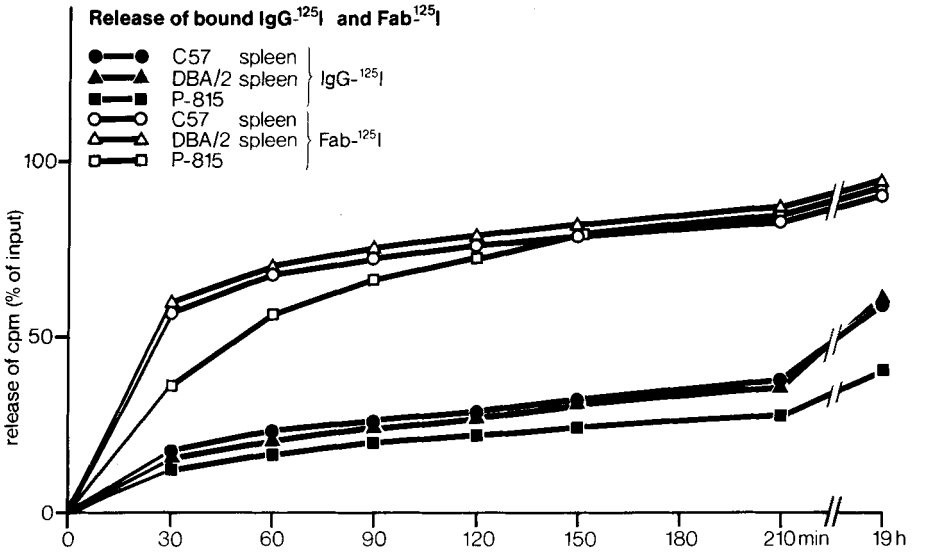


Fig. 3. Release of bound iodinated IgG anti-P-815 and Fab anti-P-815. Spleen cells or P-815 cells were incubated with the radioactive antibodies (see legend), washed and incubated at 37° C. At each time point indicated in the figure, the cells were spun down, the supernate removed and released radioactivity measured as percentage of total radioactivity present on the cells at time 0. For experimental details see also text, results section

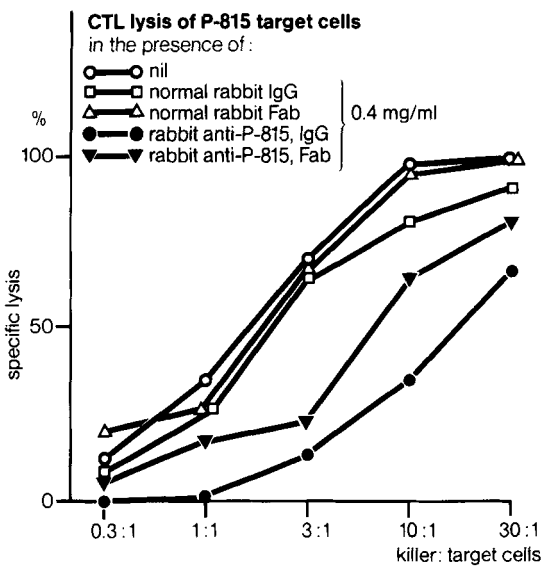


Fig. 4. Effect of normal Fab and IgG preparations on T cell-mediated cytolysis as compared with Fab and IgG preparations derived from antisera directed to P-815 cells

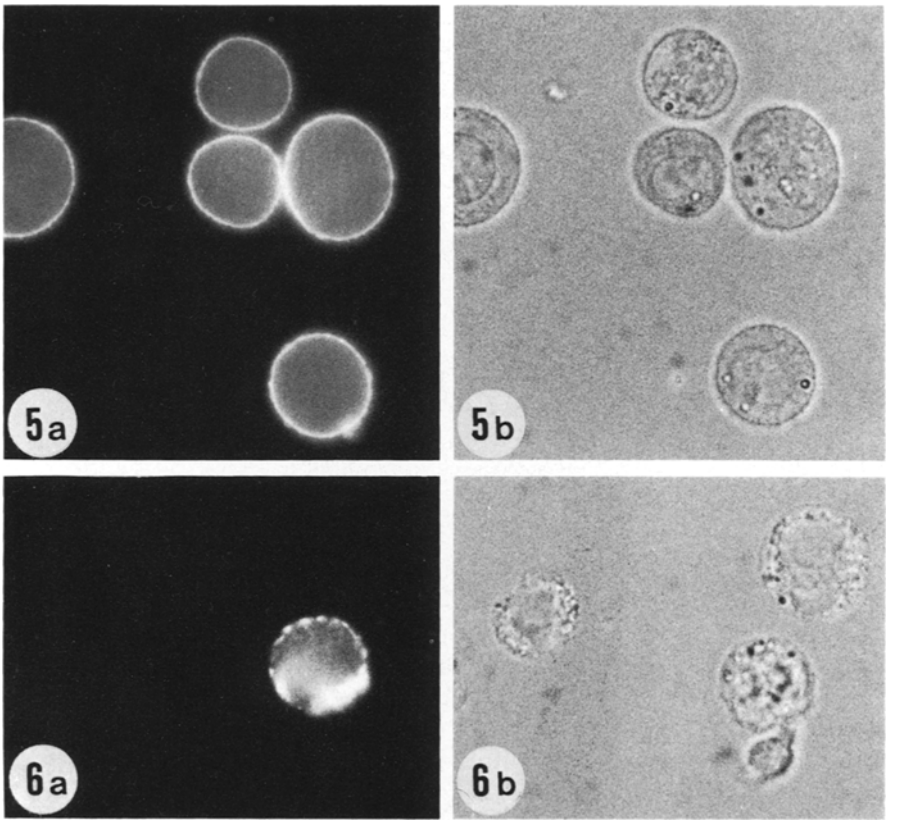


Fig. 5. P-815 cells, incubated with unabsorbed rabbit anti-P-815 antiserum, washed and incubated with a fluorescent goat anti-rabbit IgG antibody. Incubations were done at 0° C, staining is homogeneous. a, fluorescence, b, bright field. Magnification in this and all the following photographs is approximately × 800

Fig. 6. Staining of P-815 with an antiserum absorbed 16× with DBA/2 spleen cells. This antiserum was no more cytotoxic for either DBA/2 spleen or P-815 cells, but stained a minority (5–10%) of P-815 cells. DBA/2 and C57 spleen cells were not stained by this antiserum. Detection was done as in Fig. 5; a, fluorescence; b, bright field

b) T cell-mediated lysis of P-815 cells was used to demonstrate binding of IgG and Fab preparations (both on target and killer cells since all IgG and Fab preparations were prepared using unabsorbed serum). Cocultures of immune C57 spleen cells (from mice which had rejected a P-815 tumor 2–3 months earlier) with irradiated DBA/2 spleen cells were harvested, freed from dead cells on Ficoll gradients and assayed for cytotoxic activity against ^{51}Cr-labeled targets as outlined in the Material and Methods section. Each assay tube contained 0.4 mg/ml of a preparation of IgG or Fab of normal rabbit serum or of anti-P-815 antiserum or the equivalent amount of saline as control. Indeed, as Fig. 4 shows, IgG and Fab preparations derived from rabbit anti-P-815 antisera both inhibited lysis of ^{51}Cr-labeled P-815 cells by CTL while IgG

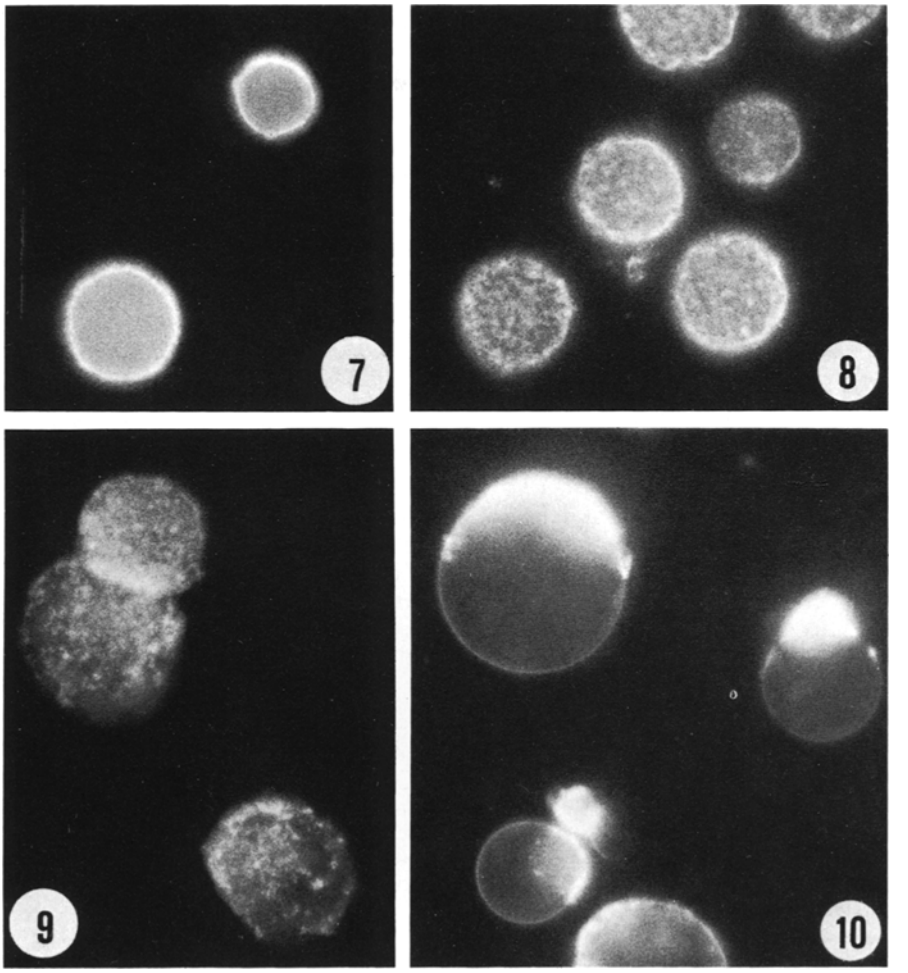


Fig. 7. P-815 cells incubated with rhodamine-coupled IgG anti P-815 at 0° C. Staining is diffuse

Fig. 8. Same cells as in Fig. 7, warmed to room temperature for 10 min. The staining pattern is patchy

Fig. 9. Same cells as in Fig. 7, warmed to room temperature for 30 min. The patches tend to coalesce at one pole of the cell but do not from true caps

Fig. 10. Same cells as in Fig. 7, warmed to 37° C for 4 h. Some cells show caps but were shown to be dead by viability tests such as trypan blue dye exclusion

and Fab preparations, derived from normal rabbit sera and used at identical concentrations, were inactive.

3. Fluorescence Results

a) Whole Anti-P-815 Antiserum, Detected by a Second Fluorescent Antibody. Samples of P-815 cells were incubated for 1 h at 0° C as outlined above with

10 or 20 µl of whole antiserum which was either unabsorbed or absorbed 16 × with DBA/2 cells. This absorbed serum had lost all cytotoxic activity towards either DBA/2, C57 or P-815 cells. After 2 washings the cells were incubated for 1 h at 0° C with a fluorescent goat anti-rabbit Ig antibody. After 2 further washings the cells were observed in suspension. Figures 5a and 6a show the fluorescent staining pattern with unabsorbed and absorbed anti-P-815 antiserum, 5b and 6b the corresponding field in ordinary light microscopy. Unabsorbed serum is able to stain all P-815 cells homogeneously, while absorbed serum stains only a small percentage (5–10%) of all P-815 cells. Since this serum does not stain any C57 or DBA/2 spleen cells, it is likely that this staining represents a tumor-specific or tumor-associated antigen. Further study should demonstrate whether the low titer observed is due to the immunization procedure and the subsequent absorptions or whether indeed only a minority of tumor cells is carrying this antigen.

b) IgG Preparations of Anti-P-815 Antiserum, Coupled to Rhodamine. Such preparations also stained P-815 homogeneously after incubation at 0° C (Fig. 7). Within minutes of observation of room temperature patch formation could be observed (Fig. 8), which after 30 min at room temperature tended to coalesce at one pole of cell (Fig. 9) without, however, forming true caps. Such caps could be observed after prolonged (2–6 h) incubation at 37° C. These caps, however, were exclusively found on dead, swollen cells (Fig. 10). It can be concluded from these experiments that P-815 cells are able to form patches quickly by bivalent ligands to their surface antigens, but that they will not form true caps under these circumstances. Using a single layer of bivalent antibody, cap formation was seen only on dead cells.

c) Fab Fragments of Anti-P-815 IgG, Coupled to Rhodamine. P-815 cells, when stained with such reagents at either 0, 20 of 37° C were all stained homogeneously. Figure 11a shows a cell suspension which had been incubated at 0° C for 2 h, washed twice, warmed up to 37° C for 1 h and observed thereafter. All cells were stained homogeneously (see Fig. 11b) for corresponding light microscopy) through the degree of staining varied considerably. Some cells developed small uropods. This did not however change the overall diffuse aspect of the staining: Cap or patch formation could not even be observed after 24 h incubation at 37° C.

d) Triple-Layered Sandwich of Fab (Anti-P-815 IgG)-Rho, Followed by IgG of Goat Anti-Rabbit IgG, Followed by IgG of Rabbit Anti-Goat IgG. In preliminary experiments it was attempted to induce patch and cap formation by a simple sandwich technique, complexing Fab fragments bound at the tumor cell surface with a second, bivalent antibody directed against the Fab fragments (IgG of

Fig. 11. Staining pattern of P-815 cells after incubation at 0° C with rhodamine-coupled Fab fragments of rabbit anti-P-815 IgG, followed by warming up for 1 h at 37° C. The staining pattern is diffuse, even when the cells form uropod-like protrusions. In this and all the following illustrations: a, fluorescence; b, corresponding area in bright field microscopy

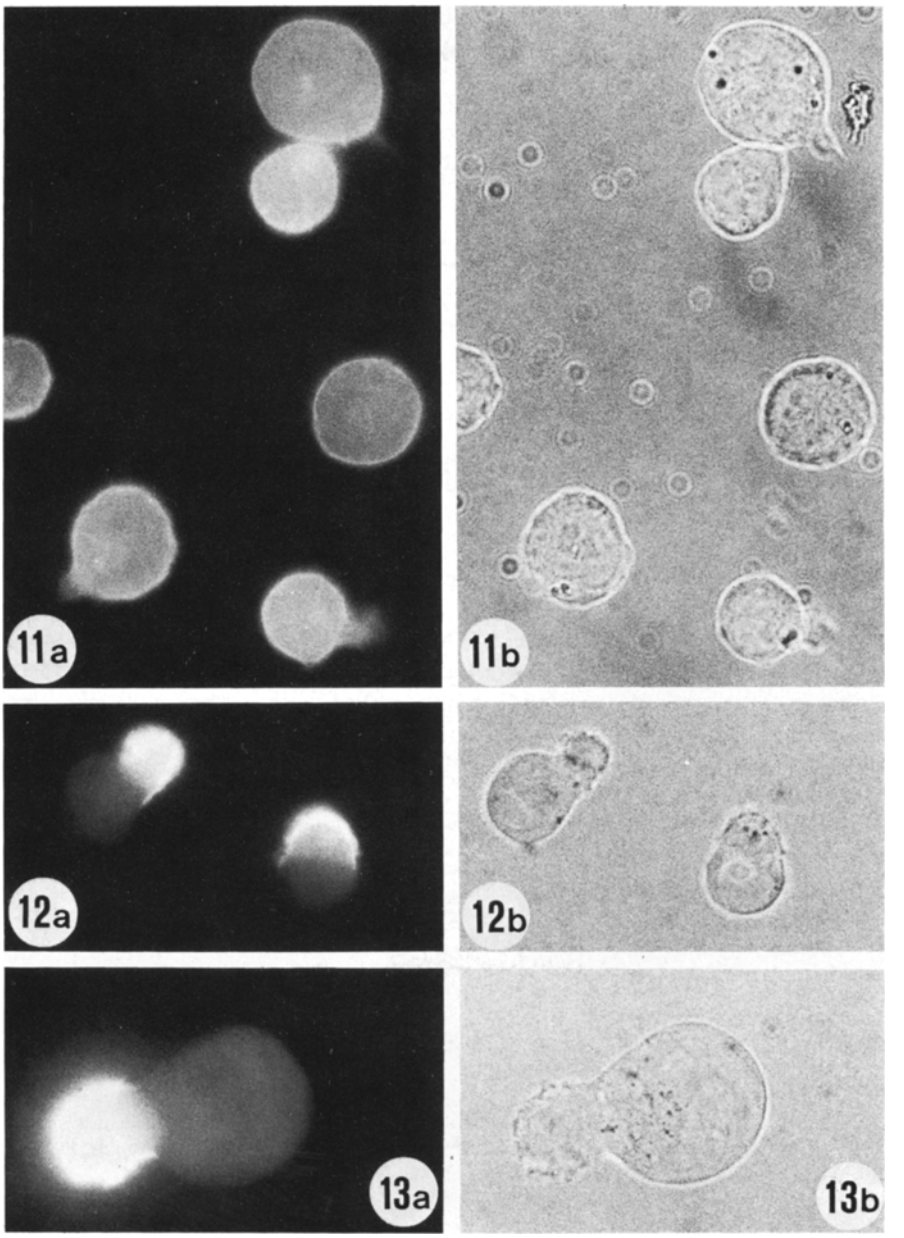


Fig. 12. P-815 cells, stained sequentially with rhodamine-coupled Fab fragments of rabbit anti-P-815 IgG, IgG of goat anti-rabbit IgG and IgG of rabbit anti-goat IgG. Incubations were done at 37° C, followed by a 1 h incubation at 37° C. Caps are present on uropod-like protrusions of the cell

Fig. 13. Same protocol as in Fig. 14. This figure illustrates particularly well the characteristics of the caps on these tumor cells, which are present on large protrusions and do not appear to be endocytosed (as are, e.g., Ig caps on the surface of B lymphocytes)

goat anti-rabbit IgG). It was indeed possible to induce patch formation by this procedure: Caps, however, appeared infrequently (less than 20%). Only a third layer of bivalent antibody (IgG of rabbit anti-goat IgG), directed against the antibody of the second layer, produced caps in more than 50% of the cells, provided that all incubations and a postincubation of 1 h had been carried out at 37° C. Figures 12a and 13a show the fluorescence of such caps, 12b and 13b demonstrate the viability of the cells by light microscopy.

e) Behaviour of Surface Antigens of Tumor Cell Targets in T Cell-Mediated Lysis as Studied by Means of Rhodamine-Coupled Fab Fragments of Rabbit Anti-P-815 IgG. In these experiments purified CTL (see Materials and Methods) were assayed for cytolytic activity in chromium release tests. In parallel tubes P-815 cells which had been labeled with rhodamine-coupled Fab fragments of rabbit anti-P-815 IgG were exposed to CTL for 15 min at 37° C at ratios of killer:targets varying between 5:1 and 10:1. After that, the pellet was gently resuspended and a sample of the suspension observed immediately. Figures 14–16 (a, fluorescence; b, corresponding field in light microscopy) show three examples of conjugates, i.e., CTL firmly attached to P-815 cells.

In our experiments with monovalent antibodies, such a capping could not be seen. During attachment as well as during the later stages of CTL-mediated lysis, the distribution of fluorescence was diffuse. Patch formation was also not obtained in more than 120 conjugates observed. A pronounced concavity appeared in cases where several killer cells attached to one target cell (Fig. 17a, b). In some cases, pieces of fluorescent material stuck to the periphery of the CTL (Figs. 18–20, a and b). Whether these pieces were left over from previous attacks or whether the presently attacked target cell formed long protrusions around the killer cell cannot be decided with certainty. The electron

Figs. 14–16. P-815 cells, stained with rhodamine-coupled Fab fragments of anti-P-815 IgG, washed, and exposed to CTL for 15 min at 37° C (for experimental details see text). The conjugates formed show a diffuse staining which is also present at the site of contact, irrespective whether one (Figs. 14, 15) or several (Fig. 16) CTL are bound to the target cell

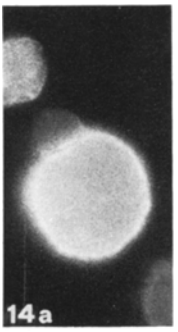
Fig. 17. Same protocol as in Figs. 14–16. Marked deformation of the target cell circumference by several adhering CTL

Figs. 18–19. Same protocol as in Figs. 14–16. Fluorescent protrusions from the target cell adhere to the CTL, forming an extended contact zone. This may be a sign of increased membrane fluidity as is observed at later stages of CTL-mediated target cell lysis

Fig. 20. Same protocol as in Figs. 14–16. Fluorescent material clinging to the circumference of CTL, possibly derived from a previous attack but probably also representing a target cell protrusion around the CTL

Fig. 21. Same protocol as in Figs. 14–16. The CTL adheres to the top of the target cell which shows blebbing of its membrane, believed to represent a sign of the final stage of target cell lysis. No redistribution of membrane antigens can be seen

Fig. 22. Same protocol as in Figs. 14–16. Further disintegration of a target cell, attacked by 2 CTL. No gross redistribution of membrane antigens can be seen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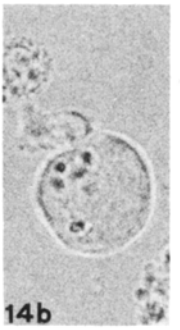
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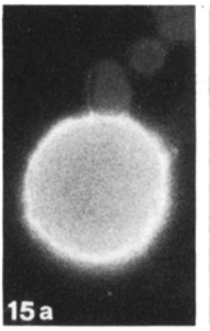
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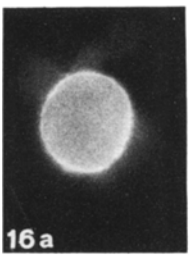
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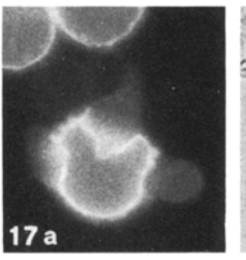
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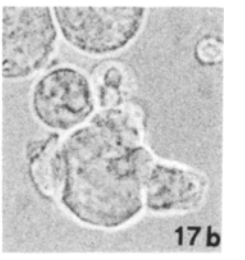
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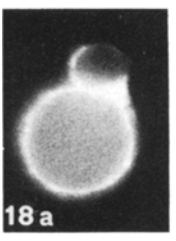
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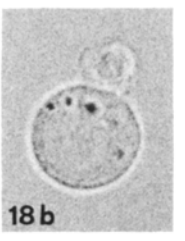
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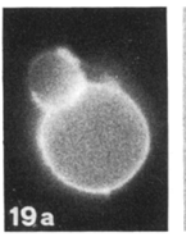
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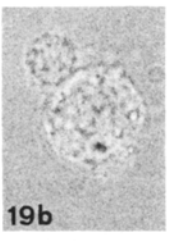
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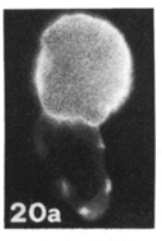
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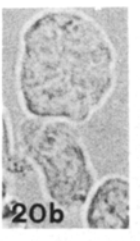
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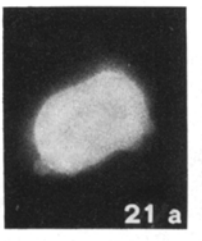
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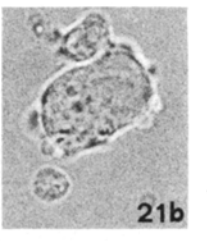
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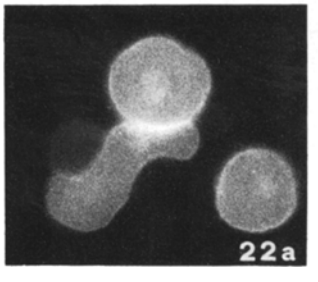
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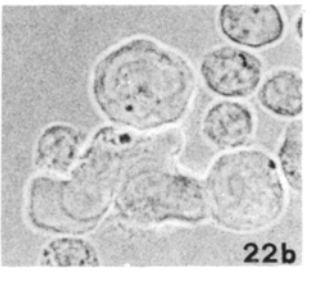
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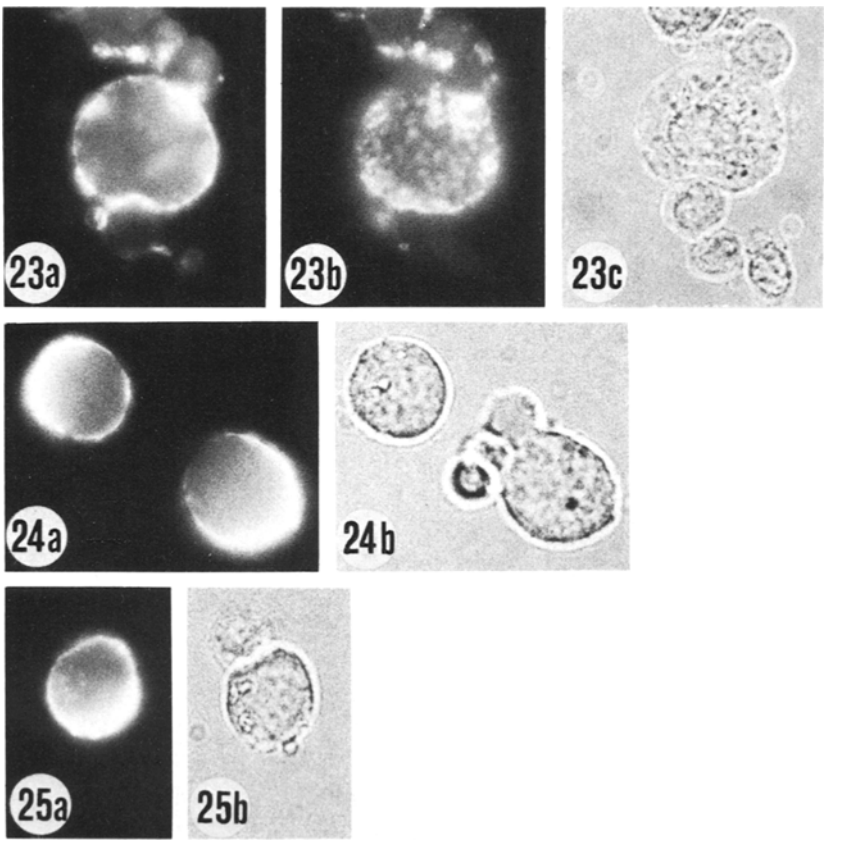

Fig. 23a–c. P-815 cells stained with rhodamine-coupled IgG of anti-P-815 antisera and exposed to CTL for 15 min at 37° C. Fluorescence (**a, b** 2 different focus levels) shows a patchy distribution which does not appear to be altered by conjugate formation; **c** bright field microscopy

Figs. 24–25. Same staining procedure as in Figs. 12 and 13, followed by an exposure to CTL for 15 min at 37° C. Two examples of CTL adhering not to the cap but to the opposite end of the target cells

microscopic evidence recently obtained (Matter 1979) would be in favor of the latter possibility. In any case, it is most unlikely that Fab fragments detaching themselves from tumor targets and rebinding to CTL would give rise to these pictures since free CTL, which were incubated with fluorescent target cells, never carried any fluorescence. Subsequent stages of CTL-mediated target cell lysis are illustrated by Figs. 21–22, blebbing (Fig. 22a, b) and a complete distortion of the cell membrane (Fig. 23a, b) are generally thought (Matter 1979; Ryser et al. 1977; Sanderson 1977) to represent late stages of target cell lysis, but did not in any way involve a redistribution of surface antigens.

f) CTL Lysis on Target Cells with Patched or Capped Surface Antigens. IgG preparations of anti-P-815 antiserum, coupled to rhodamine, were used as described above to induce patching of surface antigens of P-815 cells. Such cells

were then exposed to CTL for 15 min at 37° C and observed immediately afterwards. Figure 23a, b and c show that CTL did not induce any capping under these conditions and that the patching was random.

A triple-layered sandwich method was used as described above to induce caps of surface antigens of P-815 cells. Such cells were also exposed to CTL under identical conditions as described above. Figures 24 and 25a and b demonstrate that CTL attached to target cells were not necessarily found on top of the cap as might be expected; on the contrary, they were often (in 27 out of 43 conjugates observed) found at the opposite side of the cap. It is likely that there were enough antigenic sites left free after incubation with the antibody for the CTL to attach in a more or less random fashion to the tumor cell surface.

Discussion

Heterologous antibodies were used in this study to examine the mobility of surface antigens of tumor cells. These polyvalent antibodies recognized at least 3 different antigenic determinants: a) xenoantigens and/or public specificities common to the d and b haplotype, b) alloantigens specific for the d haplotype, and c) TATA of the P-815 cells. The latter were found only on a minority of tumor cells, probably due to insufficient strength of the antibodies (Al-Rammahy and Levy 1979; Al-Rammahy et al. 1980; Clemetson et al. 1976).

The production of pure monovalent Fab preparations was quite critical. Repeated runs on gel chromatography columns were necessary to eliminate remaining undigested IgG. In our hands, iodination of the preparations and subsequent autoradiographic analysis in SDS-gel electrophoresis proved to be the most sensitive method for evaluation of the preparations. Pure Fab preparations bound specifically to P-815 cells. Nevertheless they had a fairly low avidity which is partly explained by their monovalency, partly by the extensive shedding of surface material (Koch and Smith 1978).

The distribution of surface antigens was homogeneous. No redistribution of monovalent ligands occurred over many hours of observation irrespective of whether cells began to adhere and form uropodlike protrusions.

Bivalent ligands induced patch formation but were unable to induce capping. Caps were only obtained using a second and third layer of crosslinking reagents. Surface antigens were then found exclusively on typical uropods, while the rest of the cellular circumference appeared to be devoid of any ligand.

The fact, however, that CTL could still bind to the opposite pole of capped cells, suggests that either not all alloantigens were bound by the ligands, or, that alloantigens reappeared during incubation with CTL. A rapid modulation of H-2 antigens has indeed been described (Lesley and Hyman 1974; Loor et al. 1975). The capping induced was a slow process and only found on part of the cells. It therefore clearly belongs to the second type of capping (Braun et al. 1978; Schreiner and Unanue 1977).

The same reagents were used to examine the hypothesis that capping of surface antigens, induced by CTL, was the direct cause of death of the target cells (Berke and Fishelson 1975). Capping of H-2 antigens has been claimed

to occur at the contact site between CTL and target cells (Berke and Fishelson 1975). A limitation to this study was, however, that a sandwich method involving 2 bivalent antibodies was used and that this method by itself might have induced cap formation (Berke and Fishelson 1976; Schrader et al. 1975; Yefenor and Klein 1974). In the present study this pitfall was avoided using monovalent antibodies. Another objection might be that the antigens under study were covered by the antibody and therefore no longer accessible to CTL. Indeed, specific blocking of CTL by anti-H-2 antibody has been described by several authors (Brunner et al. 1968; Edidin and Henney 1973; Faanes and Choi 1974; Germain et al. 1975; Schrader and Edelman 1976; Todd et al. 1973). It should be recognized, therefore, that minute changes, such as "micropatching", could not be studied with the methods being used at present. The question which this study was able to answer was whether or not CTL binding to target cells induced a gross redistribution of surface antigens.

Such redistribution might represent the lethal hit for the target cell via various mechanisms:

a) transmembrane proteins might be pulled closely together to form a gap junction. Such junctions have been described by some authors (Fergula and Allison 1974; Grimm et al. 1979; Henney 1973; Sellin et al. 1971), their existence denied by others (Matter 1979; Sanderson et al. 1977),

b) ligand-receptor complex formation by the attachment of CTL to their targets might induce a diminished fluidity of the membrane with phase separations, involving mainly cholesterol. Such a process has been described for Con A, induced ligand-receptor complex formation (Zagyansky and Jard 1979) and the fact that cholesterol is crucially important for target cell lysis to occur at least when present in CTL (Heiniger et al. 1978) might be seen as an argument in favor of such an hypothesis,

c) local shear forces, brought about by attachment and subsequent contraction of the contractile network of the CTL have been proposed (Seeman 1974) as the direct cause of permeability changes. The sensitivity of CTL-lysis to cytochalasin B (Cerottini and Brunner 1972) is certainly compatible with this hypothesis,

d) finally, the direct attachment of CTL to targets might induce alterations in the membrane potential with subsequent changes in the permeability of the cell (Berke and Amos 1973).

The fact, that in our study a gross redistribution of membrane antigens could not be seen obviously does not eliminate the above hypotheses since a subtle rearrangement of a few surface antigens ("micropatching") could not be ruled out. Such subtle changes might still have important physiological consequences.

Our study is essentially in agreement with the recently published findings (Hale and Paulus 1979) that enucleated cells, with reduced capacity to cap their surface antigens, were still highly susceptible to CTL-mediated lysis.

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