LOCALIZATION AT HIGH RESOLUTION OF ANTIBODY-INDUCED MOBILIZATION OF VACCINIA VIRUS HEMAGGLUTININ AND THE MAJOR HISTOCOMPATIBILITY ANTIGENS ON THE PLASMA MEMBRANE OF INFECTED CELLS*

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Subsequent to the demonstration that the major histocompatibility molecular complex (MHC)[1] is a necessary determinant in immune specific killing by cytotoxic lymphocytes (CTL) in virus-infected target cells (1), elucidation of the phenomenon of MHC restriction has been sought through several different experimental approaches. On the basis of detailed genetic analyses (2–4) involving several different viruses and murine cells recombinant in the MHC loci or possessing a large variety of minor heritable differences, such as single amino acid changes, it was concluded that MHC restriction might require a specific association between the viral and MHC determinants so as to create "altered-self" or "neoantigens" for presentation to the matching CTL receptors. Formation of such neoantigens presumably could occur by the comingling or closely spaced association between the relevant molecules within the plasma membrane. In fact, antibody-mediated mobilization of independent determinants into common patches or caps, termed syncapping (5), has been documented clearly with vesicular stomatitis virus (VSV) and other virus-infected cells (6) by means of light microscopy of fluorochrome-labeled conjugates. Alternatively, instead of presuming that MHC and viral antigens are formed into an altered-self complex, it is quite plausible, in terms of the "dual recognition" hypothesis, that interaction of CTL with their targets is only contingent upon the close proximity at the surface of the two relevant determinants and not on their direct association, as discussed in recent reviews (7, 8). Other evidence (9–11), arising from a combination of genetic, microscopic and chemical analyses, has been interpreted to show that with

[1] *Abbreviations used in this paper*: CTL, cytotoxic lymphocytes; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; GAM5, goat-anti-mouse 5 nM gold conjugate; GAM-FITC, fluorescein-linked goat-anti-mouse conjugate; GAM-Rho, rhodamine-linked goat-anti-mouse conjugate; GAR20, goat-anti-rabbit 20 nM gold conjugate; GAR-FITC, fluorescein-linked goat-anti-rabbit; HA, hemagglutinin; HB, hybridoma antibody; MHC, major histocompatibility complex; NM, nutrient medium; PFU, plaque-forming units; VSV, vesicular stomatic virus.

the paramyxoviruses, including measles and Sendai viruses, attachment of specific antibodies does not cause the viral and MHC antigens to become concentrated into shared zones of the membrane and, therefore, that associations such as syncapping may not be required for recognition and killing by CTL.

In the case of vaccinia virus-infected cells, numerous studies have reliably documented MHC restriction in regulation of the CTL response (12–18), and other studies have characterized viral antigens expressed on the surface of infected cells (19–22), thus making this an appropriate model for studying interaction between viral and MHC receptors at the surface. The current effort to map simultaneously, at high resolution, MHC and vaccinia virus molecules was favored by a number of factors, including the availability of specific antibodies and antibody conjugates made with tracers readily identifiable by electron microscopy. The viral antigen in this study is the hemagglutinin (HA), a glycoprotein of 85,000 mol wt, which is expressed at the cell membrane as a late-late function but is not a constituent of the vaccinia virion itself (21). Recent purification and characterization of the HA made it possible to raise a monospecific antibody to this antigen (22), while identification of human and murine MHC determinants could be made by means of the relevant monoclonal antibodies. It therefore became feasible to simultaneously label HA and MHC by means of immunoelectron microscopy, using antibodies conjugated with colloidal gold particles of defined, predetermined sizes (23, 24).

Materials and Methods

Cell and Cultures.   Strain L_{929} fibroblasts, derived from an $H-2K^k$ C_3H mouse and human HeLa cells, expressing the A2, 28 HLA type, were grown as monolayers in nutrient medium (NM) consisting of Eagle's minimum essential medium supplemented with 5–10% fetal calf serum (FCS), according to published methods (25). The nonadherent, immortalized lymphoblastoid Raji cells expressing the A-3/BW35 HLA type (kindly typed by P. Terasaiki, UCLA Medical School, Los Angeles, CA) were grown in NM composed from RPMI 1640, supplemented with 5–10% FCS.

Virus, Infection, and Assay.   The hemagglutinin-positive IHD-J strain of vaccinia virus (20) able to replicate in a wide range of vertebrate cells was propogated and either partially or stringently purified and assayed for infectivity as plaque-forming units (PFU) by previously published techniques (25). In the present experiments, L_{929} and HeLa cells, after release from monolayers by trypsinization or the nonadherent Raji cells, were suspended in NM at 5×10^6 cells/ml and mixed with virus suspensions at a multiplicity of 5 PFU/cell. After adsorption for 1 h at 37°C on a roller apparatus, the cell virus complexes were washed to remove any unadsorbed virus, then suspended at $5–8 \times 10^5$ cells/ml in NM and incubated at 37°C, with agitation, on a shaker platform for 16 h.

Antibodies and Labeled Conjugates.   A clone of monoclonal hybridoma (HB) antibody CM 2.5, identifying K^kD^k of H-2, was kindly provided by Dr. Gerald N. Callahan of Scripps Clinic and Research Foundation La Jolla, CA. HB antibody of clone W6/32, recognizing the shared determinants A-, B-, and C- of HLA (26), was purchased as either culture supernatant or ascites fluid from Seralab, Crawley Down, Sussex, England. Preparation and characterization of the anti-HA monospecific polyclonal rabbit serum was recently described (21, 22). The fluorochrome-labeled preparations including fluorescein-linked goat-anti-mouse (GAM-FITC), goat-anti-rabbit (GAR-FITC) conjugates and rhodamine-linked goat-anti-mouse (GAM-Rho) conjugate were from N. L. Cappel Laboratories, Inc., Cochranville, PA. Conjugates bearing colloidal gold particles of defined sizes (23, 24), including goat-anti-rabbit 20 nM gold particles (GAR20) and goat-anti-mouse 5 nM gold particles (GAM5), were purchased from Jannsen Pharmaceutica, Beerse, Belgium.

Complement-mediated Immune Lysis.   The antibody preparations, heated when appropriate to inactivate complement (56°C for 30 min), were diluted in medium and, together with excess

concentrations of rabbit complement isolated from pooled sera, were used according to standard, published procedures (27). Specific lysis was measured by the quantity of intracellularly trapped ^{51}Cr released from prelabeled 10^5 L$_{929}$ cells in stationary cultures within 24 multi-well Linbro plates (Linbro Chemical Co., Hamden, CT). Within any experiment, samples for each treatment were prepared and assayed in quadruplicate.

Surface Labeling.   Binding of antibody and labeled conjugates was carried out as described (10, 12). For light microscopy and analysis by fluorescence-activated cell sorter (FACS), the procedures published previously (28), which are designed to ensure that saturating amounts of antibody and fluorochrome conjugates are present during absorption, were used. Labeling for electron microscopy required use of larger samples, each of 5-6 × 10^6 control or infected cells and the addition of appropriately increased volumes of the antibody-containing preparations. About 20 μl of GAM5 and GAR20 were added per sample at a final dilution of one in three.

Electron Microscopy.   After washing to remove unadsorbed conjugates labeled with gold, the cell suspensions were spun into pellets and processed for thin sectioning and electron microscopy, as described previously (29, 30).

Results

Evaluation of Specific Antibody Binding.   It was necessary, first of all, to establish that under present experimental conditions that (*a*) the antibodies were binding specifically and being applied in saturating amounts and (*b*) the concentration of the MHC antigens at the cell surface remained approximately equal in the control and infected cells. That both requirements were, essentially, fulfilled was revealed by the data from complement-mediated immune lysis, in Table I and FACS assays, exemplified in Fig. 1 a–c. In the former assay, the extent of immune lysis was dependent on the dilution of antibody applied (Table I). In the FACS analysis, the plot of frequency vs. size distribution on the left hand panels showed that infection in suspension with IHD-J vaccinia virus for 16 h did not alter appreciably the mean cell size (compare a with b).

TABLE I

Specificity of Binding of Anti-HA and Anti-MHC Antibodies Assayed by Complement-dependent Immune Lysis

Treatment		Percent ^{51}chromium released	
		Controls	Infected
Total		100.0 ± 7.1	100.0 ± 0.9
Complement alone		0.4 ± 0.0	2.5 ± 0.1
Complement + antibody to HA	1:10	4.1 ± 0.6	53.3 ± 3.2
	1:50	1.4 ± 0.1	15.9 ± 2.4
	1:250	0.7 ± 0.0	2.1 ± 0.2
Δ Complement + antibody to HA*	1:10	3.8 ± 0.5	9.4 ± 0.8
Complement + antibody to H-2K^{k}D^k			
	1:2	28.3 ± 1.7	37.4 ± 4.6
	1:10	21.9 ± 1.1	25.2 ± 0.7
	1:50	5.7 ± 1.2	7.0 ± 1.1
Δ Complement + antibody to H-2K^{k}D^k	1:2	2.0 ± 0.2	0.8 ± 0.1
Spontaneous lysis		2.5 ± 0.2	3.8 ± 0.8

* Δ heated to 56°C for 30 min.

Infected L$_{929}$ cells in stationary culture were reacted with antibody and complement according to standard procedures for measuring immune-specific lysis by the ^{51}Cr release assay (27).
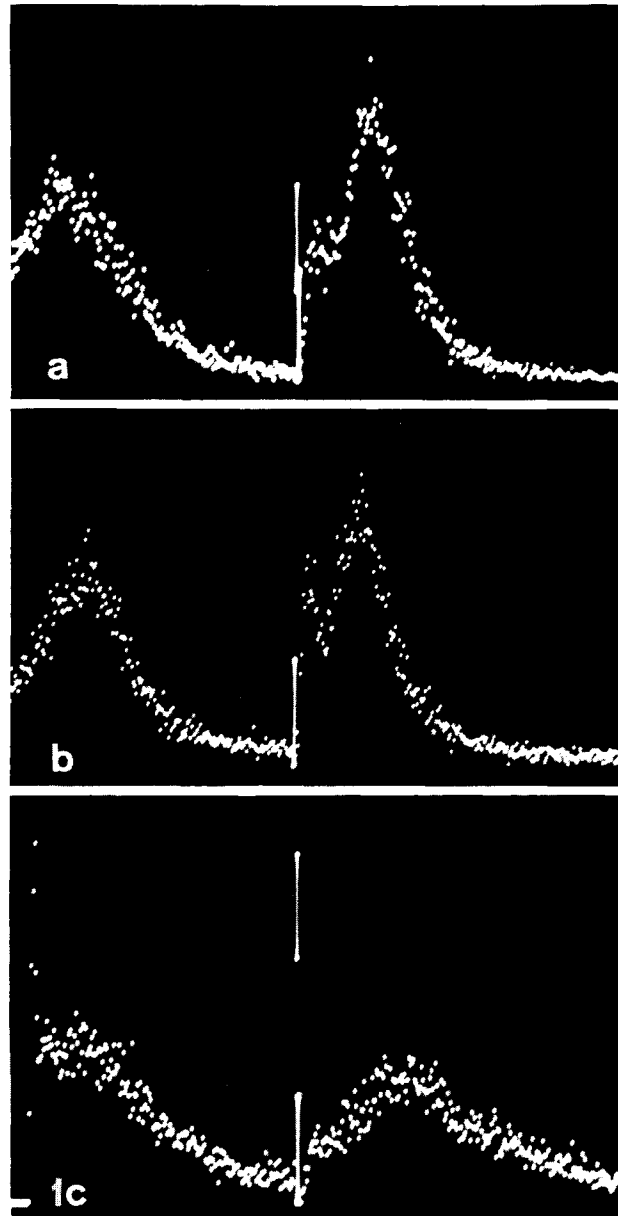
Fig. 1.   FACS measurement of the size distribution (left side of each panel) and fluorescence intensity, calibrated as described in (33) (right side of each panel) of uninfected and infected L_{929} cells. (a) uninfected, reacted with HB antibody to $H\text{-}2K^kD^k$ and tagged with GAM-FITC; (b) infected, reacted as in (a); (c) infected, reacted with antibody to HA and tagged with GAR-FITC. In each case, the size or relative intensity is plotted on the abscissa and cell number, in that class, on the ordinate. Note that both the size and intensity related to $H\text{-}2K^kD^k$ antigens are quite similar, before and after infection for 16 h. In all the electron micrographs, the MHC antigen is tagged indirectly by GAM5 (arrowheads) and the vaccinia virus HA by GAR20 (arrows). m, mitochondrion.

Measurement of the concentration of H-2 at the plasma membrane, evaluated on the basis of indirect labeling of H-2K^{k}D^k on L$_{929}$ cells, showed that fluorescence intensity was similarly distributed among the population of infected and control cells (right hand panel a vs. b of Fig. 1). The quantity of bound GAR-FITC antibody conjugate used to determine indirectly the concentration of HA at the cell surface was approximately equivalent to that of GAM-FITC, as evident by comparing the distribution of the intensity of fluorescence in the right hand panel b vs. c in Fig. 1.

Distribution of MHC and Vaccinia Virus HA in Single- and Double-labeling Experiments

LIGHT MICROSCOPY. Comparative experiments were carried out on controls and vaccinia virus-infected human Raji or HeLa and murine L$_{929}$ cells, to localize the distribution of MHC and HA antigens expressed at the cell surface. The experimental conditions were standardized with respect to the amounts of specific antibodies and fluorochrome-labeled antibodies added, duration and temperature of incubation to allow antibody binding, and occurrence of patching or capping. Usually, when labeling with two fluorochromes was undertaken, the MHC was identified by GAM-Rho and the HA by GAR-FITC.

From the series of experiments, among them those listed in Table II, the following observations were made with all three lines used as the host cells for vaccinia virus: (a) MHC and HA were uniformly distributed over the entire cell surface when specific antibodies were bound at 0°C; (b) elevation of temperature for 30 min to 37°C, or even to only 22°C, elicited prominent mobilization of antigens into patches and caps; (c) binding of single antibodies to either MHC or HA induced concentration of

TABLE II

Distribution of MHC and Vaccinia Virus HA Antigens Identified with Specific Antibodies on the Surface of Raji Cells*

Experiment number	Controls or infected‡	Temperature (0°C, 37°C) and sequence of addition of specific and labeled second antibody	Distribution of label at the cell surface	Observations by
1	C	Antibody to HA 0°C → antibody to MHC 0°C → R 0°C	R widespread	LM
2	C	Antibody to HA 0°C → F 0°C → antibody to MHC 37°C → R 0°C	R in patches or capped	LM
3	C	Antibody to MHC 37°C → antibody to HA 0°C → F + R 0°C	R in patches or capped	LM
4	INF	Antibody to HA 37°C → F 0°C → antibody to MHC 0°C → R 0°C	R, F extensive co-patching, co-capping evident	LM
5	INF	Antibody to MHC 37°C → antibody to HA 0°C → F + R 0°C	Same as experiment 4	LM
6	C	Antibody to MHC 37°C → antibody to HA 0°C → GAM5 + GAR20	Only 5 nM gold in patches and capped	EM
7	C	Antibody to MHC 0°C → antibody to HA 0°C → GAM5 + GAR20	Only 5 nM gold widespread	EM
8	INF	Antibody to MHC 37°C → GAM5 + GAR20	Only 5 nM gold in patches and capped	EM
9	INF	Antibody to HA 37°C → GAM5 + GAR20	Only 20 nM gold in patches and capped	EM
10	INF	Antibody to HA 37°C → antibody to HA 0°C → GAM5 + GAR20	5 and 20 nM gold in co-patches and co-capped	EM
11	INF	Antibody to HA 37°C + antibody to MHC 37°C → GAM5 + GAR20	5 and 20 nM gold in co-patches and co-capped	EM

* A series of comparable experiments was carried out with the same antibody to HLA-A, -B HB antibody and antibody to HA antibody using HeLa cells and with an HB-specific antibody to H-2K^{k}D^k and antibody to HA antibody using L$_{929}$ cells. Antibody to HA is rabbit serum vs. HA; Antibody to MHC is mouse HB antibody to HLA; A, B; F, goat- antibody to rabbit FITC conjugate; R, goat- antibody to mouse rhodamine conjugate; GAM5, goat- antibody to mouse 5 nM gold conjugate; GAR20, goat antibody to rabbit 20 nM gold conjugate; LM, light microscopy; EM, electron microscopy.
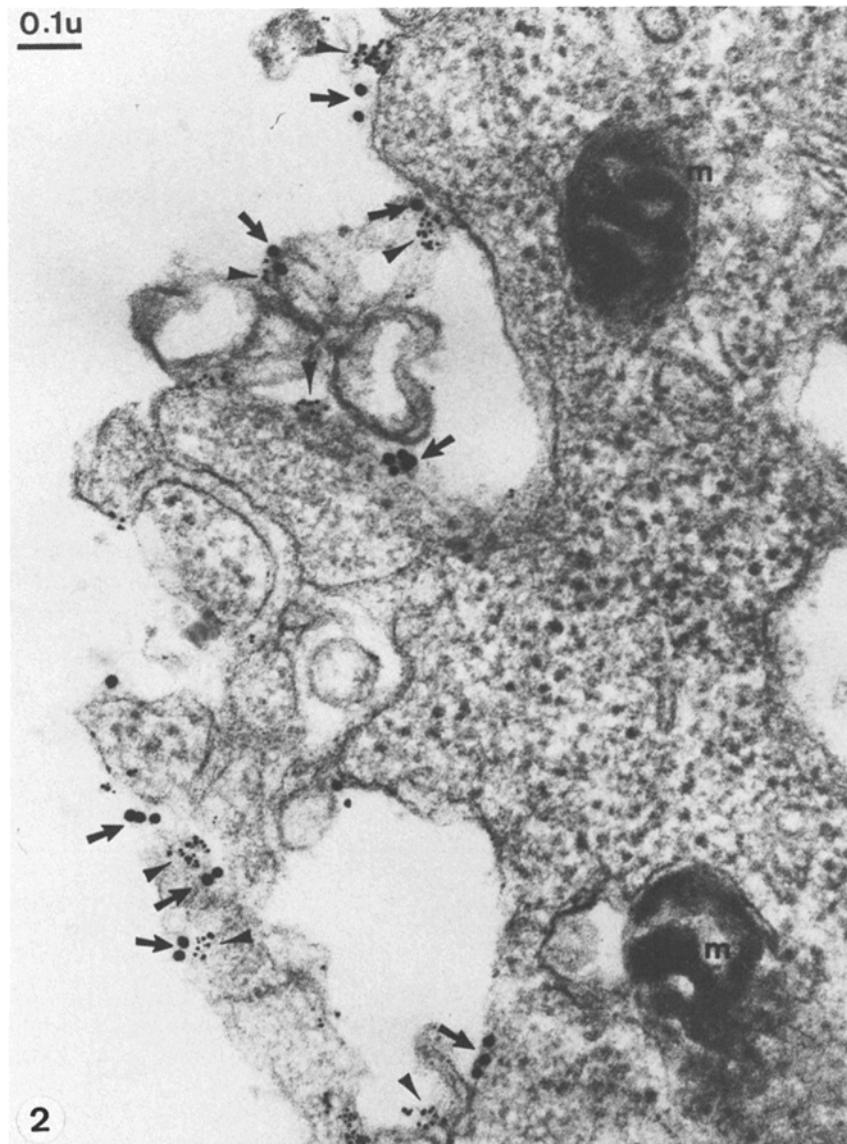‡ C, control; INF, infected.

Fig. 2. Periphery of an infected Raji cell exposed simultaneously to antibody against HLA-A, -B and antibody against HA. Note the concentration of the two antigens and their close proximity to one another at the region where there was intense activity (blebbing or capping) at the cell surface. × 97,000.

antibody molecules to both antigens within coincident patches and caps; and (d) the order in which the specific antibodies were added, whether first against MHC or HA, did not alter the coincident distribution of the fluorochrome labels. From the above observations, it may be concluded that, after antibody modulation, MHC and HA occur at surfaces of vaccinia virus-infected cells in close proximity. Therefore, upon the sequential application of specific antibody to either one of these antigens or simultaneous addition of antibodies against both antigens, MHC and HA can be
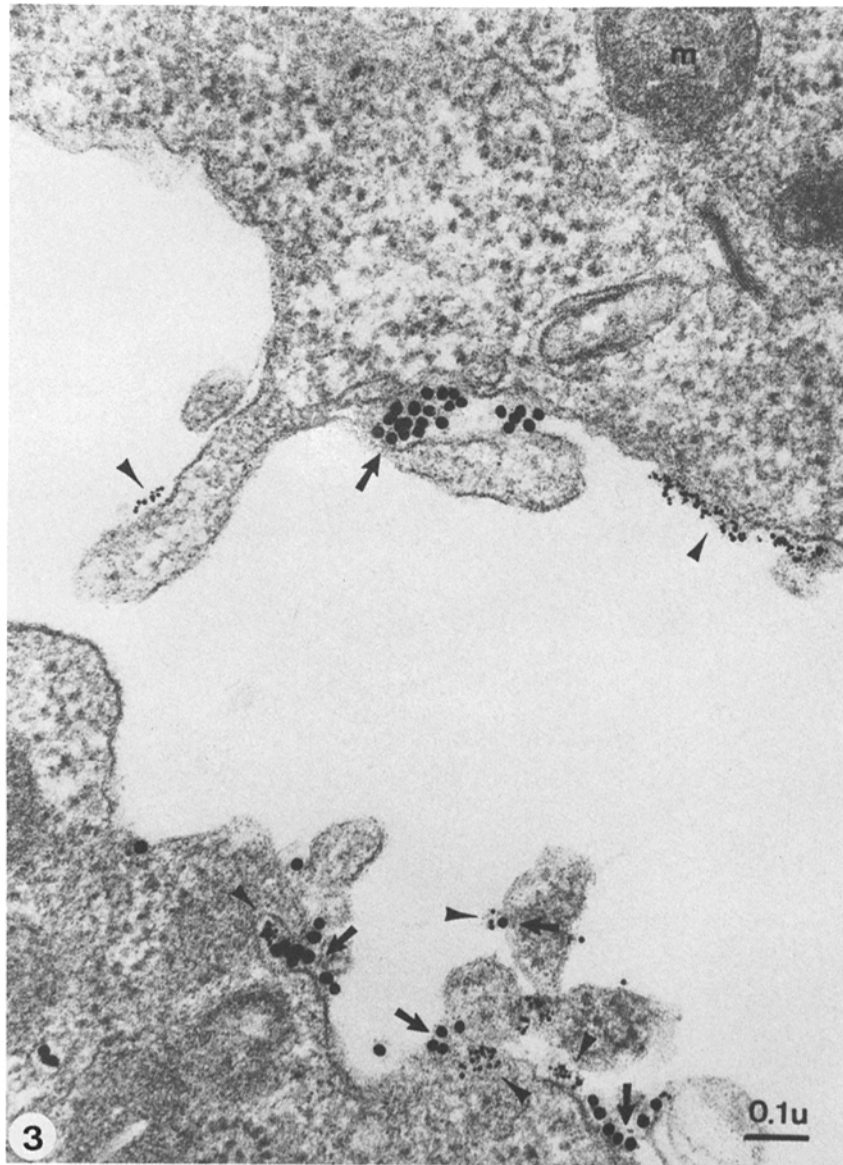
FIG. 3. Peripheral regions of two infected L_{929} cells reacted simultaneously with antibody to H-$2K^kD^k$ and antibody to HA. The two antigens are concentrated and in close proximity to one another on the lower cell but occur in separate patches at the surface of the cell above. × 97,000.

induced to co-patch or co-cap.

ELECTRON MICROSCOPY. To extend the observations obtained by light microscopy, we undertook a series of comparable labeling experiments using techniques suitable for analysis at the higher resolution of the electron microscope. For this purpose, labeling with fluorochrome antibody conjugates was replaced using conjugates consisting of dense, colloidal gold particles of predetermined, uniform size (23, 24). Use
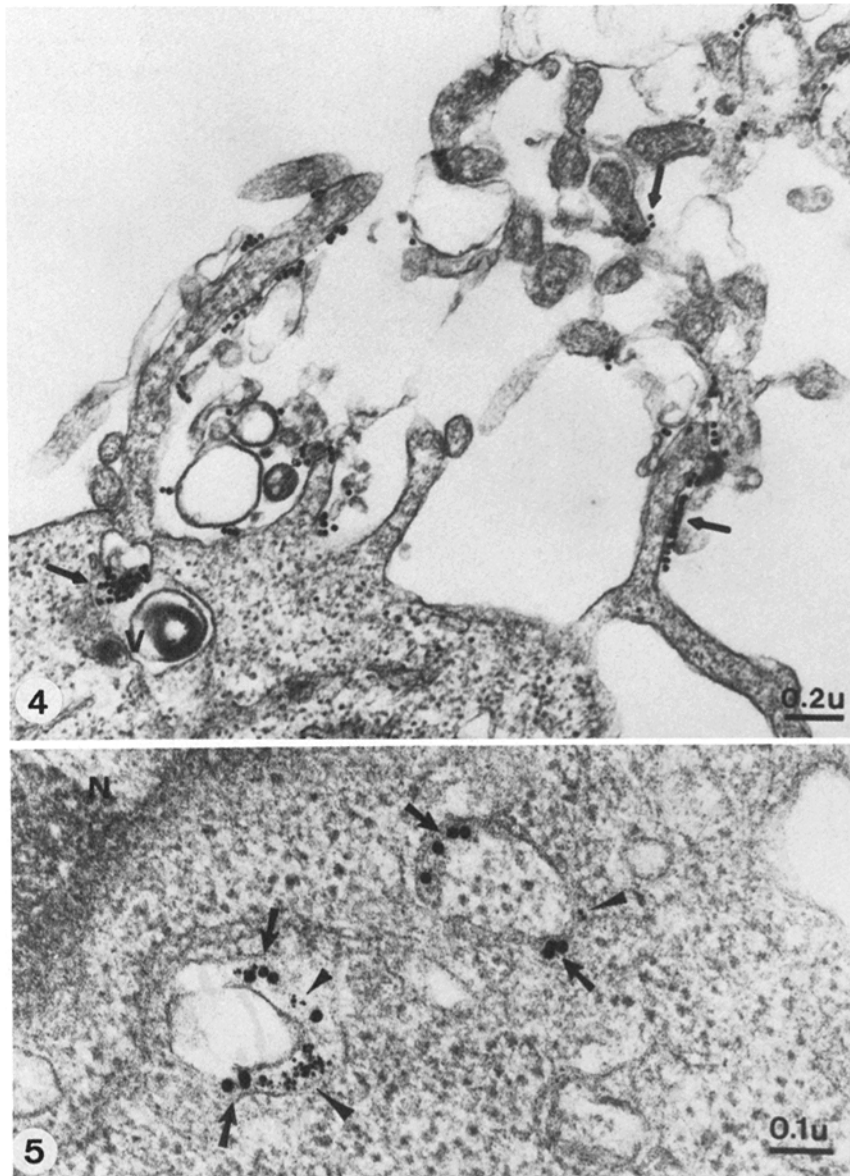
FIG. 4.   Segment of an infected Raji cell reacted only with antibody against HA but exposed to both GAM5 and GAR20 conjugates. Note complete absence of the smaller, 5 nM gold particles from the surface-active region. V, vaccinia virion. × 43,000.

FIG. 5.   Area of the peripheral cytoplasm of an infected L_{929} cell reacted with antibody to H-2K^kD^k and antibody to HA. The plasma membrane is at the extreme right. Presence of two closely associated 5 and 20 nM gold particles at the membrane surrounding the vacuoles implies that co-patched H-2 and HA antigen-antibody complexes became internalized within endocytic vacuoles. N, nucleus. × 93,000.

of such gold conjugates proved to be highly satisfactory in the current studies because the indirect labeling of MHC and HA proved to be very specific and almost entirely free from background binding of the gold conjugates.

On the basis of a series of experiments, including those summarized in Table II, it was established that GAR20 and GAM5, marking respectively vaccinia virus HA and MHC molecules, became concentrated in patches and caps containing frequently overlapping or closely associated 5 and 20 nM gold particles (Figs. 2 and 3). However, in some regions, the gold particles of one size or the other were concentrated into separate, albeit closely positioned, patches (Fig. 3). Although patching and capping were prominent even after exposure to only one of the specific antibodies, only gold particles of the size in the conjugate identifying the attached single antibody was present at the cell surface, as exemplified in Fig. 4. At the temperature(s) conducive to patching and capping, the ligand-receptor complexes presumably undergo endocytosis because endocytic vacuoles, such as those illustrated in Fig. 5, were frequently observed. It is significant that, when double labeling of infected cells was carried out, such vacuoles contained both the 5 and 20 nM gold particles. By comparison, in uninfected controls, endocytic vacuoles contained only the 5 nM particles related to the GAM5 conjugate identifying the MHC determinant.

Substitution of GAR20 with conjugates of goat-anti-rabbit antibodies coupled to ferritin, when used in single- and double-labeled experiments with GAM5, produced the same labeling patterns as those obtained with GAR20.

The combined evidence from electron microscopy is consistent with the idea that MHC and HA molecules may occur in close proximity at the surface of vaccinia virus-infected cells and can be mobilized into overlapping or contiguous aggregates under the influence of specifically binding antibodies.

Discussion

Despite the abrupt cessation of host-related translation that occurs after virus inoculation (31) and the reported reduced binding of anti-H-2 antisera by infected cells (15), present data from FACS measurements indicated that there was no appreciable reduction of surface MHC antigen concentration under the experimental conditions of infection used here. This result validated the comparisons between control and experimental cultures described in this article.

Observations of antibody-induced co-patching and capping, presented here, should be considered in terms of MHC restriction involved in CTL killing. The close proximity at surface active foci of the vaccinia virus and MHC determinants after addition of antibody might reflect the manner in which such determinants are mobilized during interaction of specific CTL and their infected targets (12, 14, 15, 17). The apparent freedom of migration of HLA or H-2 and vaccinia virus HA molecules within the plane of the cell membrane might be necessary for the function of the MHC restriction phenomenon. The model examined here should be contrasted with influenza and measles virus infections in which close association between MHC and viral antigens has not been observed (10, 32), although MHC restriction may occur (32, 33). In the case of VSV infection, binding of anti-G protein antibody caused a preferential syncapping and patching of the viral and MHC molecules on murine cells expressing the H-2K^b determinant over those with the H-2D^b determinant at their surface (6). The differential as compared with the co-mobilization of virus-specified and MHC antigens deserves, in the future, to be examined in relation to their associations with and function of the cytoskeletal proteins because capping can be influenced by substances disrupting the normal organization of actin filaments

and microtubules (7, 9). In particular, the recent demonstration (34) of an association between actin and the M protein of measles virus suggests that mobility of the external peplomers within the plane of the membrane could be controlled indirectly through interactions of the M protein with the cytoskeleton. Such an interrelationship might be maintained if, in fact, the peplomers are anchored to the M protein located subjacent to the plasma membrane. This idea is supported by the finding that submembranous concentration of actin occurs at the site of capping on lymphocytes, induced in these cells by exposure to lectins or antibody to specific surface molecules (35).

The present electron microscopic evidence does not, of course, aid in distinguishing between the validity of the altered-self (2–4, 15) and dual receptor mechanisms (8) for lysis of vaccinia virus-infected targets by specific CTL. Information is, however, available from chemical and immunological studies that suggests that the viral and MHC determinants exist as independent molecules. In the case of Sendai virus, possessing a powerful fusing factor, analysis of molecular complexes formed soon after virus inoculation in the presence of a heterobifunctional cross-linking agent, revealed that the viral glycoproteins were neither linked or very closely positioned to the MHC molecules (11). The ability of anti-H-2 alloantisera to interfere with CTL-mediated killing of vaccinia-infected targets implies that the determinant remained antigenically unchanged and perhaps autonomous (15, 17). Yet, the H-2 and virus determinants must have been contiguous because antiviral antiserum also blocked lysis by CTL, presumably because of steric hindrance to interaction between ligands and receptors (16).

The evidence presented here could also have a bearing on the establishment and topology of receptor and ligand interactions when CTL are in contact with their targets. Recently, observations by transmission electron microscopy revealed that specific CTL to influenza virus, selected by cloning, initiate extensive zones of contact with the infected macrophage target along cell processes and areas of invagination (36). The areas of these surface contacts appear to be as extensive as those in which dual concentrations of vaccinia virus HA and MHC molecules exist. It should, therefore, be quite informative to ascertain, during interactions between clonally selected CTL and their vaccinia virus-infected target molecules, whether both MHC and HA become concentrated at regions of cell-cell contact.

Summary

We examined the consequence of simultaneous or independent binding of monospecific antibody to the hemagglutinin (HA) of vaccinia virus and the A-, B- and C-determinants of $H^r A$ on HeLa or Raji cells or $K^k D^k$ determinants of H-2 on L_{929} cells. The bound antibodies were marked by goat-anti-mouse (GAM) or goat-anti-rabbit (GAR) fluorochrome conjugates suitable for light microscopy and GAM or GAR gold conjugates, used in electron microscopy. Specificity and amount of antibody adsorbed was ascertained by complement-mediated lysis of ^{51}Cr-labeled cells and by fluorescence-activated cell sorter analysis. Regardless of the order of either antibody to major histocompatibility complex (MHC) or antibody to HA addition after warming to 37°C, there was evidence by light microscopy for co-patching and co-capping of the viral and host antigens. Electron microscopic examination revealed that goat-anti-rabbit 20 nM gold conjugate and goat-anti-mouse 5 nM gold conjugate,

marking respectively the HA and MHC molecules, became concentrated in patches or caps in which the two antigens frequently overlapped or were closely associated. The contiguous MHC and HA antigens were also engulfed, as evidenced from the presence of two sizes of gold particles inside endocytic vacuoles. The significance of these observations is discussed in relation to the cytotoxic T lymphocyte-mediated killing of virus-infected targets.

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