Role of Receptor-Binding Activity of the Viral Hemagglutinin Molecule in the Presentation of Influenza Virus Antigens to Helper T Cells

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The concentration of antigen required to stimulate influenza virus-specific helper T cells was observed to be dependent upon the antigenic form bearing the relevant determinant: intact, nonreplicative virus was needed only in picomolar amounts, while denatured proteins, protein fragments, or synthetic peptides were required in micromolar concentrations for a threshold level of stimulation. Antigenic efficiency of intact virus was found to result from the attachment of virus to sialic acid residues on the surface of the antigen-presenting cell since (i) spikeless viral particles lacking the hemagglutinin molecule were much less efficient antigens for helper T cells and (ii) continuous presence of hemagglutination-inhibiting anti-hemagglutinin antibodies reduced efficiency of stimulation by intact virus ~100-fold for both hemagglutinin and internal virion proteins. Influenza virus associated rapidly with antigen-presenting cells; less than 10 min at 20°C was sufficient to introduce virus for a maximal level of T-cell stimulation. This rapid attachment was blocked by antibodies to the hemagglutinin or by pretreatment of the antigen-presenting cells with neuraminidase to remove the cellular virus receptor. Following viral adsorption by antigen-presenting cells, a lag period of 30 min at 37°C was required for the expression of helper T-cell determinants. One early event identified was the movement of the virus to a neuraminidase-insensitive compartment, which can occur at 10°C, but which was not equivalent to expression of helper T-cell determinants. Preincubation of cells with virus at 10°C for 4 h reduced the lag period of helper T-cell determinant expression to 15 min when these cells were shifted to 37°C, suggesting that transition of the virus to a neuraminidase-resistant state is a required step in presentation of T-cell antigenic determinants.

Activation of helper T cells (T_H) is required for the development of protective immunity to many viruses (9). Following influenza virus infection or immunization, T_H are needed for development of both antiviral B cells and cytotoxic T-cell responses (3, 4, 50). The antigen-specific stimulation of T_H in turn is dependent upon the ability of the antigen-presenting cell (APC) to take up free antigen and render it into a form recognized by T_H in association with major histocompatibility complex class II molecules (5). This process of antigen presentation (for review, see references 13 and 49) requires a series of events which are not yet well defined but which can be blocked by certain pharmacological agents or bypassed by various experimental manipulations (i.e., fragmentation, unfolding, or direct introduction into the membrane) (44, 47, 51).

The use of influenza virus as a model for studying viral antigen presentation has advantages, including (i) knowledge of the sequences of its component proteins (33), (ii) a relatively well-characterized infectious cycle (40, 55), and (iii) the availability of T_H clones specific for each of its major structural proteins: the envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA) and the internal components matrix protein (M) and nucleoprotein (NP) (24). Some viral determinants, recognized by certain human (32) and murine (21) T_H clones, can be mimicked by short peptide homologs of viral proteins. These alternate forms of T_H determinants can be used to distinguish properties intrinsic to the virus or to the APC that may be relevant to antigen presentation. For example, Londei et al. (36) showed that human thyrocytes aberrantly expressing class II molecules

presented antigen to anti-influenza T_H clones only if it was added in the form of a peptide containing the determinant, not as intact virus. Autologous peripheral blood lymphocytes were able to present whole virus to the same T_H , suggesting that the thyrocytes may be deficient in processing of the complex form.

We have previously noted a difference in the antigen dose response of murine T_H clones to different forms of influenza virus antigens: one group of T cells derived from immunization with whole A/PR/8/34 influenza virus was shown to respond to syngeneic APC plus antigen as the whole virus, purified HA, isolated HA1 (heavy polypeptide of HA), or a synthetic peptide of residues 111 to 119. However, the concentration of antigen necessary to elicit threshold responses varied from $<10^{-12}$ M for whole virus to 10^{-6} M for peptide (21). This was not a consequence of the T cells being derived from immunization with whole virus, since T cells elicited with only the peptide immunogen showed similarly a 10^6 -fold more efficient recognition of the virus form (22). This suggested that the effect was at the level of the APC.

Since one of the major activities of influenza virus is its capacity to bind to host cells, we investigated whether this property underlies the greater antigenic efficiency of native virus. Using cloned T-cell hybridomas specific for PR8 influenza HA and for internal proteins (23), in conjunction with the B-cell lymphoma A20 as a well-characterized cloned APC (31), we have found that the activity of the viral HA in binding host cell surface sialic acids is responsible for this effect by focusing antigen onto the APC. Once bound, the virus proceeds to a compartment insensitive to the receptor-destroying activity of NA, a step which can be accomplished at 10°C. Although entry into this NA-resistant

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state is not contemporaneous with the expression of T_H determinants by APCs, temperature shift studies suggest that it is a required step along the pathway of viral antigen presentation.

MATERIALS AND METHODS

Viruses. Influenza viruses A/PR8 [A/Puerto Rico/8/34 (H1N1)] and FM-1 [A/Fort Monmouth/1/47 (H1N1)] were grown in the allantoic cavity of embryonated hen eggs and purified from the allantoic fluid by differential centrifugation and banding in a sucrose density gradient. Virus titers were determined by hemagglutination assay as described by Fazekas de St. Groth and Webster (18), and protein determination was performed by using the Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, Calif.). Virus infectivity was destroyed by exposure to UV light (2 min at 15 cm, with a UVGLD58 Mineralight Lamp; Ultra-Violet Products, Inc., San Gabriel, Calif.) and loss of infectivity verified on MDCK cells. To prepare internally labeled virus, 0.5 mCi of [35 S]methionine (New England Nuclear Corp., Boston, Mass.) was injected into the allantoic cavity of 11-day-old embryonated hen eggs 24 h after virus inoculation. Allantoic fluid was collected 48 h thereafter, and radiolabeled virus was purified as described above.

Other antigens. Bromelain treatment of the virus resulting in the release of viral glycoproteins from the core was performed as described by Brand and Skehel (7). Preparation of HA rosettes (aggregates of HA) by treatment of whole virus with ammonium deoxycholate was as described by Laver and Webster (35). The synthetic peptide of amino acids 111 to 120 of PR8 HA1 bearing an additional cysteine at the N terminus, containing a major BALB/c mouse T_H determinant, was synthesized and, for some experiments, coupled to keyhole limpet hemocyanin as previously reported (22). The molar concentration of the peptide in the keyhole limpet hemocyanin conjugate was determined by use of a radiolabeled peptide tracer during the coupling procedure.

Antibodies. The H18-L9 (anti-HA immunoglobulin G subclass 2a [IgG2a]) and H17-D8 (anti-NP IgG2a) antibodies (54) were purified from ascites fluid by affinity chromatography on protein A-Sepharose (Pharmacia, Inc., Piscataway, N.J.). Protein concentrations were determined with the Bio-Rad dye reagent, using rat gammaglobulin (Sigma Chemical Co., St. Louis, Mo.) as a standard.

ELISA for viral antigens. Antigens were dried onto polyvinyl 96-well plates and blocked with Dulbecco phosphate-buffered saline (PBS)-5% fetal calf serum-0.5% Tween 20-0.08% NaN_3 . Viral antigens were assayed by using a mixture of monoclonal antibodies to each of the HA, NP, or M protein, followed by biotinylated 187.1 rat anti-mouse kappa chain monoclonal antibody (52, 53) and avidin peroxidase (Sigma). Final washes were done with tap water, and the chromogen 3,3',5,5'-tetramethylbenzidine was used for detection of the peroxidase (6). Plates were read on a Biotek EL309 plate reader (Biotek Instruments, Inc., Burlington, Vt.) at 450 nm, with a 750-nm reference wavelength. Known protein concentrations yielded expected dilution curves in enzyme-linked immunosorbent assays (ELISA).

Cell lines. The $H-2^d$ B-cell lymphoma line A20 1.11 was a gift of J. Kappler (National Jewish Hospital, Denver, Colo.). It was recloned and has been shown regularly to be free of mycoplasma contamination. The BALB/c ($H-2^d$) influenza-specific T-cell hybridomas used in this study have been described previously (22-24). Table 1 lists the nominal

TABLE 1. Antigen specificities of the T hybridomas used in this study^a

T hybridoma	Specificity	Restriction
P1.F12.C4	HA site 1 (HA1 111-119)	I-E
3.2B11		I-E
7/9BD5	HA site 2 (HA1 126-138)	I-A
3H10-3		I-A
1AG6	HA site 3 (HA1 302-313)	I-E
3X3D1	NP	I-A
5G5		I-A
7/9CD5	M	I-A
2LB2		I-A

^a Class II restriction was determined with L cells transfected with class II products substituted for the A20 cells in the standard assay.

antigen specificities and major histocompatibility complex restriction of T hybridomas used in this study. Major histocompatibility complex restriction was determined by stimulation of T cells by antigen plus L cells ($H-2^k$ fibroblast line) transfected with $E^d_\alpha E^d_\beta$ or $A^d_\alpha A^d_\beta$ genes (a generous gift of R. Germain, National Institutes of Health, Bethesda, Md.). A BALB/c T hybridoma specific for chicken ovalbumin, isolated in this laboratory by J. L. Hurwitz, was used as a control in certain experiments. Cells were cultured in either Iscove's modified medium (26) or RPMI 1640 (for the L cells) supplemented with glutamine and 5% fetal calf serum and incubated at 37°C in 6% CO_2 .

T-cell stimulation assay. Stimulation of T hybridomas followed essentially the method described previously (24). Briefly, microcultures (0.2 ml) containing A20 cells at 5×10^4 per well (or doubling dilutions of A20 cells if prepulsed with antigen; see below), T hybridomas at 2×10^4 per well, and dilutions of antigens were incubated for 40 to 48 h at 37°C in 6% CO_2 . Serum-free Iscove's medium supplemented with 0.05% (wt/vol) bovine serum albumin (BSA) was used throughout. Supernatants were harvested and assayed for interleukin-2 (IL-2) production, using CTLL indicator cells (20). Stimulation of CTLL cells was quantitated by the method of Mossmann (41), using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma). Briefly, after removing 80 μl of medium from each well, 8 μl of 5 mg of MTT per ml in PBS was added to CTLL cultures (0.1-ml volume) and incubated at 37°C for >4 h. Then, 0.1 ml of absolute isopropanol-0.4% HCl (vol/vol) was added to each well, and the plates were sealed with Parafilm and kept overnight at room temperature to allow the dye crystals to dissolve before reading at 570 nm (reference wavelength at 750 nm). The IL-2 assay was performed with triplicate wells and exhibited standard deviations of 5 to 9% of obtained values. The CTLL assay system was unaffected by use of non-UV-treated virus for T hybridoma stimulation.

Pulsing of APC. Washed A20 cells in serum-free Iscove's medium were mixed with virus at concentrations of 10^7 cells per ml and 10^3 hemagglutinating units (HAU) of virus per ml and incubated at room temperature for 10 min. The cells were then separated from free antigen by pelleting through 10% (wt/vol) BSA in PBS, transferred to a new tube, and washed twice in PBS and once in Iscove's medium before adding to stimulation cultures. Cell washes were performed at 4°C.

NA treatment of APC. A20 cells were suspended at 10^7 cells per ml in PBS containing 10^{-4} M CaCl_2 and 0.2 mg of NA (*Clostridium perfringens* type V; Sigma) per ml and incubated at 37°C for 60 min. Cells preexposed to virus were treated for 30 min at 37°C, using the NA at 0.4 mg/ml (39).

Cells were then washed twice with ice-cold PBS and once with Iscove's medium or binding medium (Iscove's supplemented with 0.2% BSA) before being tested.

Infection of APC. A20 cells treated with infectious virus were washed, transferred to 24-well flat-bottomed culture plates, and cultured in Iscove's medium supplemented with 1% fetal calf serum for 6 h at 37°C in 6% CO₂. The serum encouraged firm attachment of the cells to the wells in which all of the following steps were performed. The medium was changed to serum-free Iscove's medium and the cells were incubated under the same conditions for a further 16 h, followed by a PBS wash and fixation with 50% methanol–50% acetone for 3 min at room temperature. Fixed infected cells were incubated for >6 h at 4°C with dilutions of monoclonal antibody mixtures as in the ELISA. After reaction with a peroxidase-conjugated anti-mouse immunoglobulin preparation (DAKO Corp., Santa Barbara, Calif.), the stain was developed with hydrogen peroxide (1:5,000 of 30%) and dianisidine (Sigma) (1:50 from a saturated solution in 95% ethanol). The color was allowed to develop for >16 h at room temperature before counting. The percentage of cells which stained darkly (infected) was determined by counting >500 cells per well. Background staining due to surface immunoglobulin on the A20 cells proved to be at a low level.

Fixation of APC. Paraformaldehyde fixation of APC was performed by the method reported by Allen and Unanue (2) with slight modification. Antigen-pulsed A20 cells which had been centrifuged through 10% BSA were washed once with PBS and then suspended at a density of 10⁷ cells per ml in 0.5% (wt/vol) paraformaldehyde in PBS at room temperature (20 to 23°C) for 20 min. This suspension was diluted with PBS and washed twice with PBS and then with Iscove's medium. The fixed cells were incubated at 37°C for 30 min and washed twice more with Iscove's medium before being placed in T-cell stimulation cultures.

FIG. 1. Stimulatory efficiency of different forms of antigen bearing the sequence 111 to 119 of the HA1 molecule of influenza virus. A20 cells and P1.F12.C4 hybridomas were incubated with dilutions of the various antigens, and IL-2 production was measured as described in Materials and Methods. The molar concentrations of the 111 to 119 determinant in the various wells were determined by the following estimates: 10⁷ virions per HAU (16); 10³ HA molecules per virion (33); and 1,500 daltons molecular size for the peptide (21). The amount of rosetted HA was determined by ELISA, using whole virus as a reference (see Materials and Methods). Symbols: Purified UV-inactivated virus (●); intact, rosetted HA (■); 111 to 119 peptide (□); keyhole limpet hemocyanin-coupled peptide (○). O.D. 570, Optical density at 570 nm.

FIG. 2. T-helper-cell stimulating efficiencies of whole virus and bromelain-treated virus cores. Whole PR8 virus and PR8 core preparations were subjected to 10-fold dilutions and tested for their abilities to stimulate the P1.F12.C4 (HA-specific) and 3X3D1 (NP-specific) hybridomas. ELISA determined these preparations to be equivalent in amounts of NP and M and the virus to have 100-fold the amount of HA. Symbols: P1.F12.C4 with virus (●); 3X3D1 with virus (○); P1.F12.C4 with cores (■); 3X3D1 with cores (□). O.D. 570, Optical density at 570 nm.

RESULTS

Dependence of T_H stimulation efficiency upon the form of the antigen. A striking property of intact influenza virus as a T-cell antigen is the high efficiency with which it is presented to the T cells. Figure 1 shows that the concentration required for T_H stimulation depends upon the form of the antigen used. The T hybridoma P1.F12.C4, which recognizes amino acids 111 to 119 of the PR8 HA1 polypeptide (22), was incubated with a constant number of A20 cells and dilutions of various antigens which bear the 111 to 119 sequence. Although all forms of the antigen were able to induce maximum stimulation of the hybridoma, the synthetic peptide (residues 111 to 120), either by itself or coupled to keyhole limpet hemocyanin, was six to seven orders of magnitude less efficient on a molar basis than intact, noninfectious virus or the isolated, aggregated HA. It has been shown previously that the low stimulatory efficiency of the peptide is not a property only of synthetic antigens, because cyanogen bromide fragments of the HA molecule and reduced and alkylated HA also exhibit reduced stimulatory efficiencies (21). Also, the relative stimulatory efficiency of the various antigens was consistent over a wide range of APC/T-cell ratios (not illustrated). Threshold concentrations of the non-native forms of the viral antigens are comparable to those required by T hybridomas which recognize other protein antigens such as myoglobin, cytochrome, insulin, and lysozyme (14, 25, 37, 45, 48). We found no evidence of extracellular degradation of antigen as the cause for lower stimulatory efficiency: dilutions of reduced and alkylated HA were coincubated for 48 h with A20 cells and T hybridomas specific for M or NP. Supernatants from these cultures demonstrated no decrease in stimulatory ability when subsequently used as the antigen source for HA-specific T hybridomas (not shown).

Internal antigens of the virus were also presented with high efficiency when intact virus was used. This was demonstrated by comparing the stimulatory efficiencies of intact virus and virus which had been stripped of the majority of

FIG. 3. Pulsing of A20 cells with whole virus or HA-free virus cores. A20 cells were pulsed with virus or an equivalent amount (as judged by NP and M content) of virus cores for 10 min as described in Materials and Methods. The antigen-pulsed cells were subjected to twofold dilutions and tested for ability to activate T-hybridoma 3X3D1 (NP specific) (■, □) or 2LB2 (M specific) (●, ○). Closed symbols, Virus pulse; open symbols, pulse with cores. O.D. 570, Optical density at 570 nm.

the outer proteins (HA and NA) by bromelain treatment. Such virus "cores," which have greatly reduced ability to bind to cells, are much less effective in stimulating the 3X3D1 (NP-specific) T hybridoma than the intact virus (Fig. 2), although both contain the same amounts of internal antigen. The effect of denaturing the NA was also investigated. Treatment at 55°C for 19 h destroyed 95% of the viral sialidase activity while having minimal effects upon the HA titer. These preparations were as stimulatory as untreated virus (results not shown), suggesting that the viral NA activity plays no major role in the stimulatory efficiency of the intact virus.

Rapid interaction of virus with the APC was also mediated by the HA molecule: A20 cells were incubated with intact virus or virus cores (having comparable amounts of the internal proteins) for 10 min at room temperature as described in Materials and Methods and tested for antigen-presenting ability. Cells pulsed with the intact virus were stimulatory for the 3X3D1 (NP-specific) and 2LB2 (M-specific) T hybridomas at standard densities in the microwells, while those pulsed with the virus cores were not stimulatory even when used at much higher densities (Fig. 3). Thus, (i) only a brief period is required for APCs to acquire sufficient virus for maximal T_H stimulation, and (ii) the lower stimulatory efficiency of the cores when present constantly in culture is paralleled by the inability of these cores to pulse the APC in the 10-min period.

Inhibition of virus adsorption to cell surface receptors reduces its stimulatory efficiency. The observations described above suggested that binding of the HA to cell surface sialic acid residues was responsible for the high efficiency of viral antigen presentation and rapid pulsing by intact virus. If so, treatments that inhibit binding of virus to cells should reduce the efficiency of the virus to that of the core preparation or any noncytophilic protein antigen.

Attachment of the virus to the APC could be perturbed by a hemagglutination-inhibiting anti-HA antibody. This was ascertained with ^{35}S -labeled virus and various dilutions of antibody. Table 2 shows that the presence of increasing concentrations of the anti-HA monoclonal antibody H18-L9 not only reduced the number of A20 cells becoming infected,

TABLE 2. Monoclonal anti-HA antibody inhibits the binding of PR8 influenza virus to A20 cells

Concn of purified H18-L9 antibody ($\mu\text{g}/\text{ml}$)	^{35}S -labeled PR8 virus bound to A20 cells (cpm) ^a	% A20 cells expressing viral proteins ^b
0	1,804	21.6
2.6	1,758	18.6
12.8	1,188	15.5
64	187	8.9
320	118	3.0

^a ^{35}S labeled PR8 virus (4,000-cpm input) was pulsed at 0°C with A20 cells for 45 min and then washed and counted as described in Materials and Methods.

^b Infection detected by immunoperoxidase staining of A20 cells, using a mixture of monoclonal antibodies to detect viral HA and NP on infected cells.

but also decreased the amount of ^{35}S -labeled virus physically associated with the cells. For further studies, virus at various dilutions was mixed with a high concentration of antibody (giving complete hemagglutination inhibition) or PBS and added to stimulation cultures with APC and T hybridomas specific for HA or NP. Even after 48 h of coculturing, the PR8 virus-antibody mixture was two to three orders of magnitude less stimulatory than virus alone (Fig. 4), while the control virus FM-1, which failed to react with the antibody, was unaffected in its ability to stimulate in this assay (not shown). The presentation of an unrelated

FIG. 4. Decrease in efficiency of presentation by neutralizing antibody when cocultured with the virus. UV-inactivated PR8 virus was combined with the anti-HA antibody H18-L9 or the control anti-NP antibody H17-L8 to give 2×10^3 HAU of virus per ml and 1.3 mg of antibody per ml. This amount of H18-L9 was more than sufficient to block completely hemagglutination of chicken erythrocytes by PR8. The mixtures were subjected to 10-fold dilutions and plated with A20 cells and the hybridoma P1.F12.C4 (a) specific for HA or 3X3D1 (b), NP specific. Symbols: PR8 virus with no antibody (■); PR8 virus with H18-L9 (anti-HA) (●); PR8 virus with H17-L8 (anti-NP) (▲). O.D. 570, Optical density at 570 nm.

antigen, chicken ovalbumin, to an ovalbumin-specific T hybridoma was found to be unaffected by the presence of PR8 virus and neutralizing antibody, ruling out nonspecific inhibition by virus-antibody complexes (not illustrated). The NP-specific antibody H17-L8 (which does not affect virus adsorption) had no effect upon the stimulation of either the HA- or the NP-specific T hybridomas (Fig. 4). Consistent with these results, A20 cells pulsed with the antibody-neutralized virus were negligibly stimulatory for several hybridomas recognizing determinants of internal and external proteins (results not shown). Thus, at effective neutralizing concentrations of antibody, the extreme dose-response and pulsing efficiencies of the virus are significantly reduced.

Virus attachment could also be perturbed by treatment of the cells with NA, which removes the terminal sialic acid residues of the surface glycoproteins to which the viral HA binds. When the APC were pretreated with exogenous NA before exposure to virus, attachment of ^{35}S -labeled virus to A20 cells at 0°C and subsequent infection were prevented (not shown), and these cells were no longer able to be pulsed with virus for T-cell stimulation (Table 3, experiment A). Such pretreatment with NA did not diminish the ability of A20 cells to present pulsed ovalbumin to an ovalbumin-specific T hybridoma (data not shown). This suggests that the effect of the enzyme pretreatment was not due to nonspecific impairment of antigen acquisition. If the cells were pulsed first, incubated at 20°C to allow virus uptake (described in more detail below), and then treated with NA, there was no effect on either infection (not shown) or T-cell stimulation. This demonstrates that the enzyme treatment itself does not impair the ability of the APC to present antigen (Table 3, experiment B). Finally, if, instead of pulsing, virus was coincubated continuously with NA-treated A20 cells and T hybridomas, there was no effect upon T-cell stimulation (Table 3, experiment C). This is consistent with the findings of Buck and Warren (8) that re-expression of sialic acid residues occurs within 24 h following NA treatment.

Presentation of viral determinants requires movement of the virus to an NA-resistant region of the cell. Kinetic studies were performed to test if presentation of viral determinants correlated principally with the presence of virus attached to

TABLE 4. Kinetics of presentation of influenza virus to T cells at different temperatures

Time (min) of incubation of A20 cells before paraformaldehyde fixation ^a	Stimulation of T hybridoma (% of maximum) in presence of A20 cells from the following incubations ^b	
	37°C	10°C
0	0	0
15	0	ND
30	32	ND
60	71	0
240	100	0

^a A20 cells (10^7 per ml) plus 10^3 HAU of UV-inactivated PR8 virus per ml were either (i) mixed on ice 10 min at 0°C, washed, and incubated at 37°C, or (ii) mixed and left together at 10°C. At noted intervals, aliquots of cells were washed, fixed with 0.5% paraformaldehyde, and used as APCs in T-cell stimulation cultures.

^b Stimulation of HA-specific T-hybridoma P1.F12.C4 measured by IL-2 release, using CTLL indicator cells. Data were presented as percentage of the CTLL stimulation obtained with 4-h cultures (37°C) or in a parallel control at 37°C for the 10°C experiment.

the cell surface or with movement of the virus to another compartment of the APC.

The initial experiment defined the time course of T_H determinant presentation by antigen-pulsed APCs (12, 56): A20 cells were pulsed for 10 min with intact PR8 virus and then cultured for various time periods at 37°C before fixation with paraformaldehyde. Table 4 shows that a lag period of about 30 min at 37°C is required for the appearance of the HA site 1 T-cell determinant, as well as for T_H determinants on internal proteins (not shown). By contrast, incubation of APC plus virus for up to 4 h at 10°C did not result in detectable appearance of T_H determinants. These results indicate that (i) adsorption of virus to the cell surface is not itself sufficient for antigen presentation, (ii) further events on or within the APC are required for development of the T_H determinants, and (iii) these events are either drastically slowed or perhaps even blocked at 10°C.

To study the events that occurred (or were blocked) at 10°C, the following experiment was performed (Fig. 5). A20 cells were incubated at 10°C for various periods with ^{35}S -labeled virus, UV-inactivated virus, or infectious PR8 virus. At intervals, cells were separated from unbound virus by centrifuging through BSA at 4°C and either directly counted to determine the amount of cell-bound ^{35}S -labeled virus or treated with NA to remove reversibly bound virus (40). The NA-treated cells were then incubated under the appropriate culture conditions at 37°C to allow completion of infection and of antigen presentation. Figure 5a shows that ^{35}S -labeled virus achieved maximum binding to the cells at 1 h of exposure, but the spontaneous elution from cells resulted in a decline in the amount of bound virus over the remaining incubation period. However, the amount of radiolabeled virus becoming resistant to removal by NA increased as a function of time at 10°C (Fig. 5b). Paralleling that increase was a rise in the number of A20 cells becoming infected (i.e., expressing newly synthesized viral proteins) and in the ability of the cells to present T_H determinants derived from both internal and external virion proteins. These increases are not simply due to the continued accumulation of virus on the cell surface at 10°C, since virus adsorption was maximal at 1 h of exposure (Fig. 5a). Rather, it indicates that the virus that becomes resistant to removal by exogenous NA is the antigen that is relevant for subsequent antigen presentation.

That entry of the virus into an NA-insensitive compart-

TABLE 3. Abrogation of ability to rapidly introduce virus for subsequent presentation to T_H by NA pretreatment of APC

Expt	APC treatment ^a				Relative stimulation (%) of T hybridomas in presence of APC treated as indicated ^b			
	NA, pre	V, pulse	NA, post	V, plate	P1.F12	7/9BD5	5G5	2LB2
A	—	+	—	—	100	100	100	100
	+	+	—	—	0	0	0	0
B	—	+	—	—	100	100	100	100
	—	+	+	—	91	76	114	133
C	—	—	—	+	100	100	100	100
	+	—	—	+	124	133	102	80

^a A20-1.11 cells were either treated with *C. perfringens* NA (NA) or left untreated and then pulsed with whole, purified PR8 (at 10^3 HAU/ml) virus (V) for 1 h at 0°C (experiments A and B) or cultured in the presence of 10^3 HAU of virus per ml (experiment C). For experiment B, NA post-pulsing treatment followed a 1-h incubation at 20°C to allow for virus uptake after the pulse.

^b Microcultures containing treated A20-1.11 cells in serial twofold dilutions and T hybridomas were assayed for IL-2 secretion as outlined in Materials and Methods. Stimulation is presented as a percentage of the response obtained with nonenzyme-treated control APCs for each experiment.

FIG. 5. Correlation of capacity of virus-exposed APCs to subsequently present viral antigens with development of an NA-resistant association of virus with cells. A20 cells were incubated with ^{35}S -labeled PR8 virus, infectious nonlabeled virus, or noninfectious UV-inactivated virus at 10°C . (a) Total ^{35}S -labeled virus associated with cells was determined by washing aliquots of cells at various intervals and measuring bound radioactivity ($\bullet$), without enzyme treatment of cells. (b) At intervals, virus-exposed cells were washed and treated with *C. perfringens* NA (30 min, 37°C) to remove virus attached to cell surface sialic acid. Cells were then counted to measure NA-resistant ^{35}S -labeled virus ($\bullet$), cultured at 37°C to allow expression of newly synthesized viral antigens detected by immunoperoxidase staining as a measure of infection ($\blacksquare$), or used as APCs in stimulation cultures (see Materials and Methods) with T-hybridoma 3.2B11 (HA specific; $\circ$), 3X3D1 (NP specific; $\square$), or 2LB2 (M specific; $\triangle$). Data in (b) are presented as a percentage of the maximum values obtained at the 4-h time point. The abscissas represent identical time points in (a) and (b).

ment of the APC is an obligatory step in formation and expression of viral T-cell determinants is further suggested by the following experiment (Fig. 6). A20 cells were incubated with UV-inactivated virus at 10°C . At various time points, cells were washed free of unbound virus and fixed with paraformaldehyde either immediately or at various intervals following further incubation at 37°C . As noted above, virus-treated A20 cells shifted to 37°C without preincubation at 10°C exhibited the typical 30-min lag period prior to expression of T-cell determinants, and virus-treated cells kept for up to 4 h at 10°C failed to stimulate T cells if fixed before the temperature shift. By contrast, the cells kept for 4 h at 10°C and fixed after the shift to 37°C exhibited a shortened lag period of only 15 min. Further, the degree of the T-cell response obtained at the 15-min time point at 37°C increased in proportion to the amount of time the cells were kept at 10°C . From this we infer that events occurring during the 10°C incubation, including entry into an NA-insensitive compartment, are directly relevant and required for subsequent antigen presentation.

DISCUSSION

The ability of influenza virus to attach to the cellular receptors on the APC surface appears to be responsible for the highly efficient presentation of its antigens to T_H by APCs. Low concentrations of intact virus introduce efficiently both glycoproteins and internal virion antigens. Rosetted purified HA also is an antigen able to stimulate at low molar concentrations, but HA-free virus particles produced by bromelain digestion must be present in a higher concentration to achieve equivalent stimulation of internal protein-specific T_H compared with whole virus.

In addition, it is clear that the binding of virus to the cell is itself not sufficient to effect presentation: kinetic studies show that (i) the effectiveness of antigen presentation correlates with viral uptake rather than the amount of virus adsorbed to the external cell surface (i.e., that removable by exogenous NA) and (ii) a lag period of about 30 min at 37°C is required before the bound virus is converted to a form recognizable by T cells.

The existence of a lag period following pulsing with antigen has been previously reported by Chesnut et al. (12), using ovalbumin, and Ziegler and Unanue (56) for the bacterium *Listeria*, indicating that it is a general feature of the presentation of complex antigens. As in this study, disruption of cellular function by chemical fixation or lysosomotropic agents during this lag period abrogated the expression of T-cell determinants.

However, a new observation in this research is that we were able to decrease the length of the required lag period by pretreating the APCs with virus for various times at 10°C . T-cell stimulation was then obtained at only 15 min after shifting to 37°C . Furthermore, the magnitude of the T-cell response obtained at 15-min exposure to the higher temperature was proportional to the duration of preincubation at 10°C . This was not correlated with the continued accumulation of virus on the cell surface but rather paralleled the movement of virus into the NA-resistant state, suggesting that virus is accumulating in a post-binding compartment from which it is more rapidly expressed on the APC surface

FIG. 6. Antigen presentation accelerated as a function of virus-cell interaction at 10°C . A20 cells were mixed with UV-inactivated PR8 virus at 2×10^3 HAU/ml on ice, and the mixture was incubated at 10°C for 0 ($\circ$), 1 ($\square$), 2 ($\triangle$), or 4 ($\bullet$) h. Cells were then separated from free virus by centrifugation through 10% BSA and transferred to 37°C . At the times indicated on the abscissa, aliquots of cells were fixed with paraformaldehyde and used as APCs for stimulation of the HA-specific T-hybridoma P1.F12.C4. IL-2 secretion was measured in the CTLL assay and is expressed as optical density at 570 nm (O.D. 570).

in higher amounts. At this time it seems that this step could be endocytosis of the virus, which should occur at 10°C (46), but the physical location of the virus entering the functionally defined NA-insensitive compartment has not been identified.

The exact events which occur subsequent to the entry of the virus into this NA-insensitive compartment are not yet known. From knowledge of the influenza virus infectious pathway (40, 55), and the fate of endocytic vesicles (46), events likely to ensue after uptake include fusion of the virus into the endosomal membrane and fusion of endosomes with lysosomes. It has been reported that temperatures below 20°C prevent fusion of internal vesicles in hepatocytes (17); if this is a general requirement, it may underlie the requirement for the time at higher temperature to complete the antigen presentation sequence. Allen et al. (1) also observed a reduction in release of catabolized material from ingested *Listeria* spp. at 20°C, compared with 37°C, after internalization in macrophages, an event correlated with antigen presentation. Further studies are required to define the cellular compartments relevant to antigen presentation. It will be particularly interesting to examine the role of HA-mediated fusion of viral and cellular membranes in these processes.

It should be emphasized that intact HA rather than replication of the virus is correlated with the efficiency of presentation; UV-inactivated (nonreplicative) virus is equally well presented (19). By impairing the cell-binding ability of the virus by pretreatment of target cells with NA or using anti-HA monoclonal antibodies able to prevent virus attachment, virus lost completely its ability to pulse cells. Our finding that presentation of viral antigens could be inhibited by antibodies directed to the HA would explain previous observations by different investigators that pretreatment of virus with antibodies to the HA, but not to internal virion proteins, resulted in decreased T-cell proliferation to the antibody-treated virus (10, 29). This inhibition was interpreted as indicating that T-cell responses were HA specific. It seems clear now that this effect is related to the initial virus-APC interaction which can affect presentation of both external and internal viral antigens; there is no evidence for antibodies to HA blocking T_H epitopes expressed on the APC (19) nor inhibition by monoclonal anti-NP antibody.

Although antibody to the HA decreased the antigenic efficiency of the virus, we were unable by this means to reduce its stimulatory ability to that of the peptide form of the antigen. A 3-log₁₀ decline was all that we obtained, not the approximately 6-log₁₀ difference typical when the antigen is provided as peptide. One possible explanation is that not all of the HA on the virion is blocked by antibody, and over the course of the culture (~48 h) interaction with cellular receptors may occur. Also, although we observed no evidence for Fc-mediated uptake of neutralized virus in our system [i.e., F(ab')₂ was as effective as IgG in inhibiting the virus (unpublished data)], it is possible that immune complexes are handled more efficiently than free noncytophilic antigens (11). It is also possible that, although uptake of the virus by specific receptor was blocked, pinocytosed virus may retain certain activity (i.e., HA-mediated fusion) which may once again set apart native virus from short peptides in efficiency of antigen presentation.

The general observations of this research are in good agreement with the findings of others that specific focusing of antigen onto APCs greatly increases the efficiency of antigen presentation. Using murine T cells specific for rabbit IgG, Kakiuchi et al. (28) were able to effectively pulse syngeneic B-cell APCs with a 10⁴-fold-lower concentration

of antigen if rabbit anti-mouse IgG was used instead of normal rabbit IgG. Antigen covalently coupled to anti-immunoglobulin showed enhanced antigenic potency and immunogenicity (30), probably as a result of targeting to B cells mediating an antigen-presenting function. B cells were also shown to efficiently present antigens for which their surface immunoglobulins were specific (34, 38, 42).

Since most cell types possess receptors for influenza virus, our results suggest that a varied profile of antigen-presenting cells could be involved in immune responses to influenza or presumably to any virus with affinity for receptors on potential APCs. Depending upon the requirements of the responding T cells for IL-1 or other factors (15, 27, 49) and the ability of the cell to process virus (36, 43), many Ia-bearing cells, including resting or activated B cells, could probably present viral antigens efficiently. It remains to be investigated if certain APC types preferentially stimulate T cells of different activity or specificity. Since presentation of antigen by a range of different APCs may be a normal occurrence in virus infection, use of vaccines consisting of denatured components or peptides lacking cytophilic properties may alter the profile of APCs functioning in the immune response.

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