IMMUNOLOGICALLY RELEVANT PEPTIDE ANTIGEN EXISTS ON THE PRESENTING CELL IN A MANNER ACCESSIBLE TO MACROMOLECULES IN SOLUTION

By KEMP B. CEASE, GAIL BUCKENMEYER, IRA BERKOWER, JENA YORK-JOLLEY, AND JAY A. BERZOFSKY

From the Metabolism Branch, National Cancer Institute, National Institutes of Health, and the Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20892

An understanding at the molecular level of the events involved in antigen-specific T cell activation has been a primary but elusive goal of modern immunology. The critical initial event involves T cell receptor–associative recognition of appropriately processed antigen and a specific Ia molecule at the antigen-presenting cell (APC)/T cell interface. However, direct demonstration of antigen on the cell surface has not been straightforward (1–5). If antigen were in fact present on the presenting cell surface and bound to the Ia molecule or the external surface of the cell membrane before binding to the T cell receptor, one would expect it to be freely accessible to macromolecules in solution. Yet, paradoxically, it has been notoriously difficult to block antigen presentation using antibodies specific for the antigen, in contrast to antibodies to the Ia molecule (4–8). Though a few cases of blocking with antipeptide antibodies have been reported (9, 10), others have not been able to detect such blocking in similar experiments (7, 8). Even in the former two cases, it was not possible to determine the mechanism and site of the blocking. Thus questions of antigen's existence on the presenting cell surface have not been resolved by studies of antibody blocking. Other recent studies using aldehyde-modified presenting cells suggest that relevant antigen should be present in some form on the cell surface (11) but provide no insight into its form or location.

We reasoned that many antibodies made to peptides in solution or to native protein may not bind the conformation of the peptide when it is bound to the presenting cells with sufficient affinity to block presentation. Therefore, in the current study we use the high-affinity avidin-biotin interaction to demonstrate cell surface inhibition of antigen presentation to T cells. Biotin was specifically conjugated to a site on the peptide close to that recognized by the T cell but far enough away to avoid interfering with activity for T cell stimulation. These studies show that, under physiologic conditions, immunologically relevant antigen is present on the presenting cell in a manner that is both freely accessible to large macromolecules in solution and necessary for antigen-specific T cell activation. Interpreted in the context of structural information currently available for participants in the ternary complex, these findings hold implications for models of antigen presentation for T cell recognition.

Materials and Methods

Mice. Mice used in these studies were 6- to 12-wk-old B10.D2 mice (The Jackson Laboratories, Bar Harbor, ME) or B10.GD bred by us from breeding stock kindly provided by Dr. Donald Shreffler, St. Louis, MO.

T Cell Clones. T cell clones were derived from sperm whale myoglobin–specific T cells from B10.D2 mice and maintained as described previously (12). Clone 14.1 is specific for the epitope centered around lysine 140 of myoglobin in association with I-E^d. This site was originally identified and studied in bulk cell populations and subsequently in this and other T cell clones using natural sequence-variant myoglobins, cleavage peptides, and synthetic peptides (13–15). Clone 9.27 is specific for an epitope in the region of glutamic acid 109 in association with I-A^d (12). Characterization of this site has similarly been performed in bulk and cloned populations using variant myoglobins, fragments, and synthetic peptides (13, 15, 16).

Antigens. Sperm whale myoglobin was prepared as described previously (12). Synthetic peptide 132–146 of sperm whale myoglobin was synthesized by manual solid-phase peptide synthesis as described previously (14). The trifluoroacetic acid (TFA)[1]-protected lysine derivative was selected for use in the synthesis to enable selective amino-terminal labeling of the completed peptide (17). Thus, after cleavage of the peptide from the resin by anhydrous hydrogen fluoride, the ϵ-amino groups of lysines 133, 140, and 145 remained blocked by the TFA group. We refer to this peptide as 132–146(TFA). Protein concentration of 132–146(TFA) was estimated by the method of Bradford using a BSA standard (18).

Preparation of N-α-biotinyl-132–146. ~500 nmol of 132–146(TFA) was biotinylated using 12.5 μmol (4.3 mg) of N-hydroxysuccinimide (NHS)-biotin (Pierce Chemical Co., Rockford, IL) and 12.5 μmol (5.5 mg) of sulfo-NHS-biotin (Pierce Chemical Co.) in 0.05 M borate buffer, pH 9, in 50% dimethyl formamide (DMF) for 16 h at room temperature with stirring. The reaction mixture was then chromatographed on a Bio-Gel (Bio-Rad Laboratories, Richmond, CA) P2 gel filtration column (1.5 × 80 cm) in 50% DMF to separate peptide from unreacted reagents. Discrete peaks were collected, lyophilized, dissolved in 1 M piperidine, blanketed under nitrogen, and incubated at 4°C overnight with stirring to remove TFA blocking groups. Samples were then lyophilized, redissolved in 9% formic acid, and chromatographed on a Bio-Gel P4 gel filtration column × 90 cm). The resulting fractions were qualitatively analyzed for the presence of biotin by spotting on filter paper and developing with the ureido ring–specific reagent diethylaminocinnamaldehyde (DACA) (19). Biotin-containing fractions were then assayed for specific antigenic activity in a T cell proliferation assay as described below.

HPLC Separation and Avidin Binding Analysis. Selected gel filtration peaks were further characterized by reversed-phase HPLC using a Waters Associates (Milford, MA) system and a SynChropak RP-P C18 column (SynChrom, Inc., Linden, IN) and a water, 0.1% TFA/acetonitrile, 0.1% TFA solvent system. Gradient elution at 1 ml/min was typically used starting at 100% water/TFA and increasing acetonitrile/TFA by 0.66%/min. Elution was monitored at 214 and 280 nm.

The presence or absence of biotin in resolved peaks was determined by specific adsorption by avidin-agarose. The sample to be analyzed was mixed with avidin-agarose (Sigma Chemical Co., St. Louis, MO) containing 20 nmol of biotin-binding capacity in a total volume of 200 μl. After incubation for 30 min at room temperature with occasional mixing, the avidin-agarose was sedimented by centrifugation in a table-top centrifuge. The supernatant was filtered (0.45 μm) and analyzed by HPLC as above. A parallel incubation was performed containing 100 nmol D-biotin (Sigma Chemical Co.), added before addition of peptide as a control.

T Cell Proliferation Assay. Assays were performed using complete medium consisting of RPMI 1640 with 44% Eagle-Hanks' amino acid medium, 10% FCS, 5 × 10^{-5} M 2-ME, 2 mM fresh-frozen L-glutamine (Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) as described previously (12). APC were prepared from spleens

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[1] *Abbreviations used in this paper:* DMF, dimethyl formamide; NHS, N-hydroxysuccinimide; TFA, trifluoroacetic acid.

of unimmunized mice by mashing, unit gravity sedimentation of debris, hypotonic lysis of red cells, washing, and irradiation with 3,300 rads from a ^{137}Cs source. The standard T cell proliferation assay was performed by coculturing 4×10^5 APC with 10^4 T cells in the presence or absence of antigen and/or avidin in 96-well plates with 0.2 ml total volume per well. After 72 h, wells were pulsed with 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) and harvested 16 h later onto glass fiber disks using a semiautomated device (Skatron, Sterling, VA). The radioactivity on each disc was determined by liquid scintillation counting using a Beckman LS2800 scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Assays were performed in triplicate and the geometric means and standard errors of the means were determined. Variations of this standard protocol were employed as outlined in the figure legends.

Results

Preparation of N-α-biotinyl-132–146. After biotinylation of synthetic peptide 132–146(TFA), chromatography on Bio-Gel P2 yielded three discrete peaks designated I, II, and III. This step was performed to separate peptide from unreacted biotinylating reagent before deblocking of the ε-amino groups of lysines 133, 140, and 145 so as to avoid biotinylation at these sites. After deblocking, chromatography of I, II, and III on Bio-Gel P4 yielded several incompletely resolved peaks in each case numbered with Arabic numerals according to elution time. Qualitative analysis for biotin was positive for peaks I.1, I.2, I.3, II.6, and II.7. HPLC analysis of peak I.1 yielded three peaks with retention times of 39.5, 47.5, and 52.6 min with relative areas of 1.52, 2.15, and 1.00, respectively. Total specific adsorption of peaks 47.5 and 52.6 by avidin-agarose was observed, indicating that all of the molecules in these peaks contained biotinyl groups.

Activity of N-α-biotinyl-132–146. The gel filtration chromatographic fractions containing biotin were tested for specific T cell antigenic activity for clone 14.1. As shown in Fig. 1, activity was predominantly found in peak I.1 with lesser activity in peak I.2 and none in peaks I.3, II.6, and II.7. Peak I.1 had no stimulatory activity for clone 9.27 possessing an irrelevant specificity (data not shown). The HPLC resolved peaks from I.1 were tested with clone 14.1, and only peak 52.6 was active. Thus, a single HPLC peak from I.1 was active and biotinylated. Peak I.1 material was titered for specific antigenic activity for clone 14.1, and the lowest concentration giving maximal proliferation was used in subsequent functional studies. This material possessed the expected structural and functional properties of N-α-biotinyl–132–146, and will be referred to as B132.

Avidin Inhibits Presentation of B132. Proliferation of T cell clone 14.1 was assayed with APC and antigen (myoglobin, 132–146, or B132) added 90 min before T cells with or without the addition of avidin (5 μM final concentration) 90 min before, concurrent with, or 90 min after the addition of T cells (Fig. 2) to examine the effectiveness of avidin addition at different times relative to T cell addition. Marked (90%) inhibition of the response to B132 was observed with avidin added 90 min before T cells, but only slight inhibition of the response to myoglobin and 132–146 was seen. The kinetics of inhibition appeared to be rapid relative to the rate of T cell activation, because concurrent addition gave 60% inhibition, and addition after 90 min of culture with T cells still yielded 50% inhibition. A standard 60-min interval between avidin addition and T cell
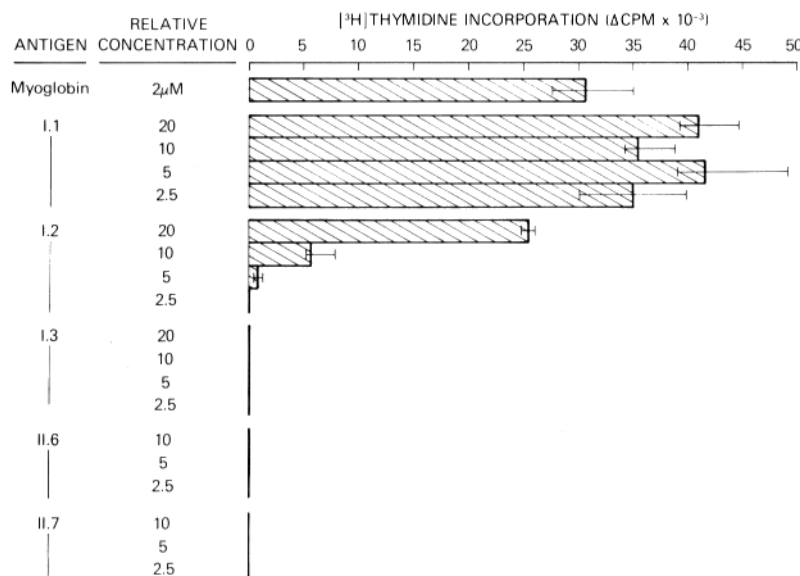
FIGURE 1. Antigenic activity of chromatographic peaks of biotinylated peptide. After gel filtration chromatography, peaks (numbered as in Materials and Methods) were assayed for their ability to stimulate clone 14.1 in the standard T cell proliferation assay as described under Materials and Methods. T cell [³H]thymidine incorporation is shown expressed as the background subtracted geometric mean cpm (Δcpm) with the error bars indicating the range for SEM for triplicate wells. The no-antigen background was 310 cpm.

addition was adopted for subsequent experiments. The effectiveness of avidin addition at varying times relative to antigen B132 is explored in the pulse/chase experiments described below.

Avidin Specifically Inhibits Presentation by APC Prepulsed with B132. Inhibition of presentation in the standard T cell proliferation assay could occur at sites of antigen uptake, intracellular trafficking, or presentation to the T cell. To rule out the possibility of inhibition occurring before or during antigen uptake or association with the APC, we used antigen pretreatment or pulsing. As shown in Fig. 3, presentation of B132 to clone 14.1 by prepulsed APC was clearly inhibited by avidin at a dose that had no effect on the presentation of the myoglobin control. The 5 μM avidin dose was found to be optimal for obtaining specific inhibition, without the nonspecific inhibition seen at 5- to 25-fold higher concentrations. In addition, this study demonstrates that full inhibition is seen even when avidin is added at a time when free antigen has been removed and any uptake of antigen by the APC that is going to occur is already complete. Thus, avidin blocking is not merely blocking of uptake by the cell.

Avidin Inhibits Presentation by Pulsed and Chased APC. Pulsing intervals of up to 8 h were examined and found to show specifically inhibitable antigen presentation, though the maximal proliferation seen with B132 tended to be lower than that seen in the standard (nonpulsed) assay protocol. Presentation of peptide 132–146 to these T cell clones has been shown not to require proteolytic processing (20). To exclude the possibility that the avidin inhibition of B132 presentation was occurring before or during some other critical processing step,
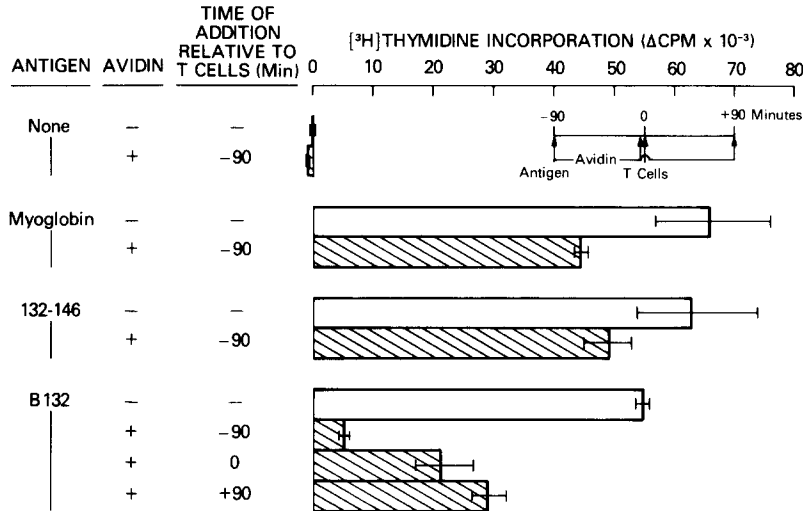
FIGURE 2.   Avidin inhibition of presentation of biotinylated peptide. The ability of avidin to specifically inhibit presentation of the biotinylated peptide was studied in the standard T cell proliferation assay and the effect of time of addition of avidin relative to T cells was also examined. In the control groups (no antigen, myoglobin, peptide 132-146), avidin was added 90 min before clone 14.1 T cells. In the group testing the biotinylated peptide B132, avidin addition was either 90 min before, concurrent with, or 90 min after addition of T cells. The inset diagram summarizes protocol timing. T cell [³H]thymidine incorporation is shown expressed as the background subtracted geometric mean cpm (Δcpm) with the error bars indicating the range for SEM for triplicate wells. The no-antigen background was 1,588 cpm.
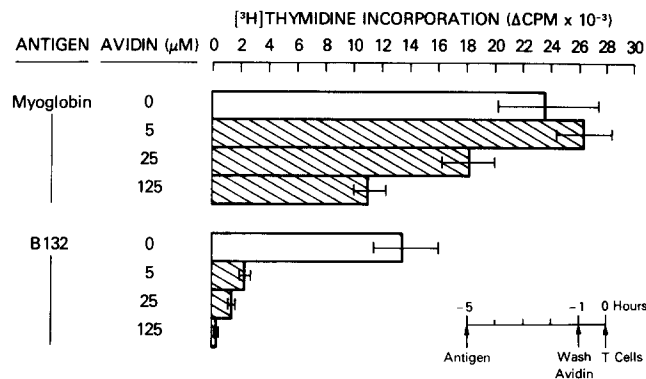

FIGURE 3.   Simple pulsing protocol and titration of avidin for inhibition of presentation of B132. APC (5 × 10⁶ cells/ml) were pulsed with antigen for 4 h at 37°C with occasional mixing. Cells were then irradiated, washed three times, and plated in 96-well plates at 4 × 10⁵ cells/well in 0.1 ml. Varying doses of avidin were added in 0.05 ml followed by 10⁴ clone 14.1 T cells in 0.05 ml 1 h later. The final concentrations of avidin are shown for each group. The inset diagram summarizes protocol timing. T cell [³H]thymidine incorporation is shown expressed as the background subtracted geometric mean cpm (Δcpm) with the error bars indicating the range for SEM for triplicate wells. The no-antigen background was 206 cpm.

experiments were performed with a chase interval of up to 16 h between the end of an 8-h pulse period and the addition of avidin and T cells. Fig. 4 shows the specific inhibition seen in such an experiment with 8 h of pulsing, followed by washing and 8 h of chase. Specific inhibition is also clearly seen with 8 h of
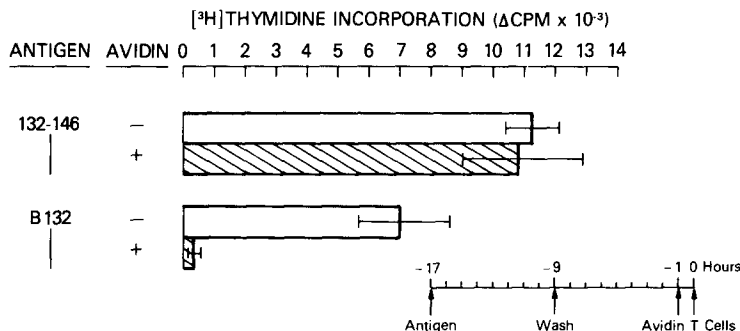
[³H]THYMIDINE INCORPORATION (ΔCPM x 10⁻³)

FIGURE 4. Avidin inhibits presentation of biotin peptide even after a culture interval (chase) between pulsing and addition of avidin and T cells. APC were pulsed as described in the legend to Fig. 4 except that a pulsing period of 8 h was used, followed by irradiation, washing, and plating. A further 8-h culture interval at 37°C was allowed before addition of avidin to allow possible intracellular trafficking of antigen to be completed, followed by addition of clone 14.1 T cells 1 h later. The inset diagram summarizes protocol timing. T cell [³H] thymidine incorporation is shown expressed as the background subtracted geometric mean cpm (Δcpm) with the error bars indicating the range for SEM for triplicate wells. The no-antigen background was 225 cpm.
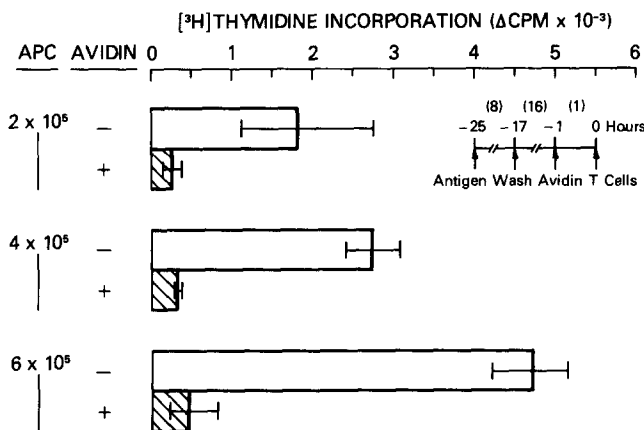
[³H]THYMIDINE INCORPORATION (ΔCPM x 10⁻³)

FIGURE 5. Increasing APC density does not diminish avidin inhibition. APC were pulsed as described in the legend to Fig. 4 except that a pulsing period of 8 h was used, followed by irradiation, washing, and plating at 2×10^5, 4×10^5, or 6×10^5 cells/well. A 16-h chase culture interval was allowed before addition of avidin followed by T cells 1 h later. The inset diagram summarizes protocol timing. T cell [³H]thymidine incorporation is shown expressed as the background subtracted geometric mean cpm (Δcpm) with the error bars indicating the range for SEM for triplicate wells. The no-antigen backgrounds were 406, 558, and 653 cpm for 2×10^5, 4×10^5, and 6×10^5 APC/well, respectively.

pulsing and 16 h of chase (Fig. 5). Therefore, avidin inhibition can occur after all intracellular trafficking that may be necessary is completed, so that inhibition cannot be attributed to blocking or altering this trafficking. The magnitude of the T cell response to B132 tended to be lower after the chase interval but still demonstrated highly significant responses with stimulation indices of 10–50-fold over the low backgrounds of a few hundred counts per minute obtained with the T cell clone. It was postulated that this might be due to a lower affinity of B132 for structures associated with the APC and consequent dissociation of some

TABLE I

Antigen Mixing Study

Avidin	Antigen	
	132–146	132–146 + B132
μM	Δcpm	
0	39,693	37,338
	(1.05)	(1.14)
20	57,118	53,669
	(1.05)	(1.12)
40	46,670	43,359
	(1.02)	(1.14)

APCs were pulsed with peptide 132–146 (2 μM) or the combination of 132–146 and B132 for 8 h, irradiated, washed, and plated. After a 16-h culture interval (chase), avidin was added to the final concentrations shown, followed 1 h later by T cells. The 4-d T cell [^{3}H]thymidine incorporation is shown for each group expressed as the background subtracted geometric mean cpm (Δcpm) with the SE factor for triplicate wells shown in parentheses. The no-antigen background was 557 cpm.

antigen during this period. If this were the case, increasing numbers of APC might provide additional peptide antigen–binding sites and thereby partially compensate for the lower B132 affinity and increase the T cell proliferative response. As shown in Fig. 5, this appeared to be the case. The interpretation that there is a greater amount of immunologically relevant antigen with increasing APC number in the B132 cultures is further supported by the finding that the response remains almost totally inhibitable. The possibility that B132-pulsed cells were more sensitive to nonspecific avidin toxicity is excluded in Table I. Including this concentration of B132 mixed with 132–146 in a pulse/chase experiment did not result in inhibition by avidin.

Avidin Inhibition Is Not Dependent on Intracellular Trafficking of Antigen. As an alternative means of determining whether intracellular trafficking of antigen was required for effective inhibition by avidin, APC pulsing was performed in the presence of monensin (Fig. 6). Monensin has been shown, at this dose, to inhibit endocytic and secretory activities of cells and recycling of receptors, class I and II MHC antigens, and surface immunoglobulin (21–26; B. Pernis, personal communication). The control response to native myoglobin was appropriately abrogated by the monensin treatment, as had been demonstrated previously (20). This control indicated that trafficking necessary for processing was effectively inhibited. However, addition of monensin to APC 30 min before and during 2 h of antigen pulsing had no effect on the T cell response to the peptide antigen B132, an indication that monensin was not toxic and that biotin–peptide did not need such processing. Under these conditions, in which intracellular trafficking was blocked by monensin, avidin still completely and specifically blocked the response to B132. Thus, avidin inhibition is not dependent on intracellular trafficking or reexpression of internal antigen and therefore must occur at some other site. The pulsing, pulse/chase, and monensin experiments together strongly suggest that blocking by avidin is occurring at the cell surface.

Antigens on APC Do Not Dissociate and Reassociate to Reform Immunologically
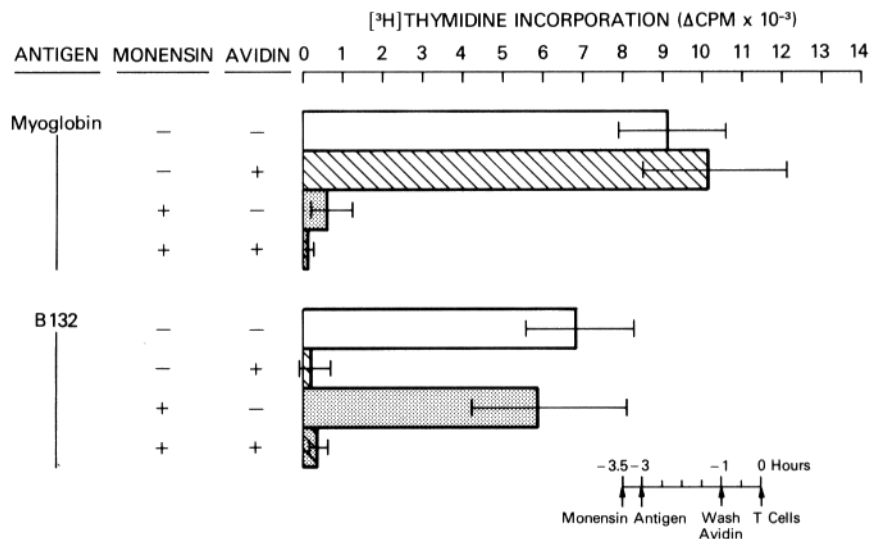
FIGURE 6. Monensin inhibition of intracellular trafficking does not affect avidin inhibition of antigen presentation. APC were incubated with or without 30 µM monensin 30 min before and during 2 h of antigen pulsing. Cells were then irradiated, washed three times, and plated as described above. Avidin additions were made, followed by clone 14.1 T cells 1 h later. The inset diagram summarizes protocol timing. T cell [³H]thymidine incorporation is shown expressed as the background subtracted geometric mean cpm (Δcpm) with the error bars indicating the range for SEM for triplicate wells. The no-antigen backgrounds were 840 and 457 cpm in the absence and presence of monensin, respectively.

Relevant Antigen. If antigen were constantly dissociating and reassociating with the APC, one might postulate that avidin could block presentation by binding to dissociated B132 before it could reassociate with the APC. We reasoned that antigen dissociating into the solution phase may reassociate with the original cell or a different cell and thus, if an antigen-pulsed cell were cocultured with a nonpulsed cell, eventually an equilibrium would be reached with equal amounts of antigen on both cells. Thus, to test this dissociation/association hypothesis, we pulsed B10.GD APC with myoglobin, 132–146, or B132 and cocultured them with nonpulsed B10.D2 APC in a T cell proliferation assay with clone 14.1. The B10.GD cells do not express an I-E^d molecule and, thus, the only way that activation of the I-E^d-restricted clone could occur would be if antigen were to dissociate from the B10.GD cells and reassociate with the B10.D2 cells in a manner that forms immunologically relevent antigen. As is shown in Table II, proliferation was not observed. Thus, such dissociation and reassociation was not detectable. Therefore, avidin blocking is not due to inhibition of such hypothetical reassociation.

Discussion

The presence of immunologically relevant antigen on the cell surface of the APC has been central to most models of antigen presentation to T cells (27–29). The initial inability to block presentation with antigen-specific antibodies prompted alternative models that allowed for concealment of antigen with exposure induced only through interaction with the T cell (4, 30). Though

TABLE II

Antigen Dissociation/association Study: Coculture of Antigen-pulsed and -nonpulsed I-E^d-positive or -negative APC in T Cell Proliferation Assay

APC		Antigen			
Pulsed	Nonpulsed	None	Myoglobin	132–146	B132
		cpm			
D2	D2	222	8,605	96,411	14,564
		(1.05)	(1.43)	(1.18)	(1.29)
D2	GD	371	8,171	66,265	13,839
		(1.23)	(1.12)	(1.03)	(1.17)
GD	D2	371	205	195	284
		(1.23)	(1.27)	(1.04)	(1.48)
GD	GD	243	229	220	419
		(1.28)	(1.18)	(1.17)	(1.06)

T cell–depleted B10.D2 and B10.GD cells were pulsed with antigens or medium (nonpulsed) for 2 h, followed by irradiation, washing three times, and plating 2 × 10^5 pulsed with 2 × 10^5 nonpulsed APC in the standard T cell proliferation assay. T cell [^{3}H]thymidine incorporation is shown for each group expressed as the geometric mean cpm (*cpm*) with the SE factor for triplicate wells shown in parentheses.

blocking T cell recognition of surface-haptenated APC by antibodies could be demonstrated, effective blocking of presentation of exogenous soluble antigen has only been demonstrated more recently and has been successful in only a few (9, 10, 31) of several well-designed attempts (4–8) to date.

The studies of Lamb et al. (9) show marked inhibition using antipeptide rabbit serum and peptide-specific T cell clones. Inhibition was not seen in an uncloned line. Corradin et al. (10), using monoclonal antipeptide antibodies, were able to demonstrate inhibition for both T cell clones and T cell lines. Though the pulsing experiments in these two studies effectively rule out blocking of antigen uptake by the APC as the mechanism of inhibition, potential blocking at other sites was not explored. In neither case was there a chase period between antigen pulse and antibody addition. Malek and Shevach (32) have shown that, immediately after pulsing, a significant intracellular antigen pool is present, whereas by 24 h after washing, almost all cell-associated antigen is associated with the cell surface. Consequently, a brief exposure to blocking antibody after antigen pulsing, as employed by Lamb et al. (9) would be expected to result in durable blocking only if antibody were taken up by the APC and were eventually able to block presentation of the intracellular antigen pool either intracellularly or extracellularly. In the pulse experiment of Corradin et al. (10), one could postulate either ongoing surface blocking by antibody or antibody uptake leading to intracellular blocking.

Surface blocking was suggested in the study of Shevach et al. (31), though in a somewhat indirect manner. Their approach was to pulse cells with exogenous antigen, allow a minimum period for antigen processing, modify the cell surface with TNP, and assay for the ability of anti-TNP antibodies to inhibit presentation to T cells. They observed inhibition in cases where the antigen contained lysine groups with ε-amino groups available for TNP modification but not when lysine-free antigens were used.

Other studies, however, have failed to detect blocking of presentation by antibodies to the antigen, including antibodies to the antigenic peptide (4–8). Therefore, the conflicting results using antibodies have not allowed a resolution of the question of antigen on the cell surface. We reasoned that perhaps the antibodies made to peptides in solution generally have insufficient affinity for the conformation assumed by the peptide on the presenting cell. In the present study we attempted to develop an experimental system that would avoid many of the difficulties of previous approaches and would allow a clear molecular interpretation of results. An antigen probe was constructed by site-specific biotinylation of a peptide with well-characterized structure/activity properties for antigen-specific activation of a T cell clone (14). This enabled high-affinity binding of avidin to the antigen regardless of its conformation and thereby avoided potential problems of concealed determinants and low affinity possible with antibodies. Specific inhibition of antigen presentation by avidin was observed in the standard T cell proliferation assay as well as under conditions designed to require cell surface inhibition.

A simple pulse protocol using 8 h of antigen pulsing was used to rule out the possibility that blocking of antigen uptake was the mechanism of inhibition. The possibility that the mechanism of inhibition was through intracellular blocking was addressed by two types of studies, providing in one case for completion of intracellular antigen trafficking, and in the other case for ablation of such trafficking. Careful studies of antigen processing have shown that immunologically relevant antigen becomes available to the T cell receptor after a minimum processing period of ~1 h (32). The reexpression of internalized antigen appears to be relatively complete by 24 h (32). Thus, after 24 h of pulse/chase, strong specific blocking of presentation by avidin would require cell surface or extracellular blocking. Clear blocking was seen in such experiments. The alternative approach was to use the carboxylic ionophore monensin to interfere with intracellular traffic in the APC. Previous studies in this laboratory have shown that monensin abrogates the presentation of native myoglobin, while presentation of peptide 132–146 is unaffected (20). These results are confirmed in the controls in Fig. 6. Formally identical results have been found in other antigen systems (11, 33). The ability of monensin to inhibit both endocytic and secretory activities of cells has been demonstrated in a variety of experimental systems and supports its use to inhibit intracellular trafficking and reexpression in studies of antigen presentation (21–23). Particularly relevant is the observation that monensin abolishes recycling of class I and II MHC antigens and surface immunoglobulin (24–26; B. Pernis, personal communication). In the current study, specifically inhibitable stimulation by B132 was seen after antigen pulsing in the presence of monensin. This indicates that intracellular trafficking and reexpression of antigen was not necessary for inhibition and thus that the critical blocking event could not be intracellular. We were unable to obtain reproducible antigen presentation to untransformed T cell clones by glutaraldehyde-fixed APC. Thus, we did not employ this additional approach in these studies. Taken together, the pulse/chase and monensin studies provide strong independent evidence that the blocking is not intracellular.

Because blocking appeared not to be occurring before uptake or intracellularly,

we proposed two remaining potential sites of blocking, in extracellular solution during transient dissociation, or at the cell surface. If the flux of antigen off of and back onto APC were great enough, one could imagine that avidin blocks presentation by binding to solution-phase antigen and preventing reassociation. To determine the magnitude of antigen dissociation and reassociation, we antigen-pulsed B10.GD APC, which do not express an I-E^d molecule and hence cannot present antigen to clone 14.1, and cocultured these cells with nonpulsed B10.D2 APC in a T cell proliferation assay with clone 14.1. No antigen presentation was seen. Therefore, the amount of antigen dissociating from the B10.GD cells and associating with the B10.D2 cells to form immunologically relevant antigen was insignificant and, thus, avidin inhibition by binding to dissociated antigen was improbable. Thus, we conclude that the blocking event is occurring at the site of immunologically relevant antigen on the APC cell surface, and that such antigen is present on the surface in a form accessible to a molecule the size of avidin.

There are three possible sites for antigen on the cell surface consistent with ultimate coordinate recognition of antigen and Ia by the T cell receptor. Watts et al. (34) and Walden et al. (35) have shown that a lipid membrane, the appropriate Ia molecule, and antigen are sufficient elements for generating a functional receptor stimulation complex for the T cell. Thus, (a) antigen could be directly associated with some part of the Ia forming the stimulation complex for subsequent binding; (b) antigen could be stably bound to both Ia and the lipid membrane at their junction to form the stimulation complex; or (c) antigen could be free in the lipid membrane in equilibrium with a low-affinity transient complex with Ia, which may be stabilized by T cell receptor binding. The third model provides for a large reservoir of antigenic peptide that could be dynamically sampled by the Ia and/or the T cell receptor. In fact, one may speculate that one reason for the immunodominance of amphipathic peptides (36) may be a positive selection based on the ability to associate stably with the cell membrane. A large pool of antigen at a high local concentration in the membrane may be necessary to provide a sufficient number of antigen–Ia complexes, given the relatively low affinity of peptide binding to Ia (37). Indeed, Watts et al. (38) have recently presented data from fluorescence energy transfer studies showing that the presence of the T cell receptor significantly increases the amount of antigen associated with Ia, possibly by stabilizing a weak complex of antigen and Ia. On the other hand, experiments using antigen-pulsed and glutaraldehyde-fixed presenting cells suggest that there are a significant number of crosslinkable antigen–Ia complexes before interaction with the T cell receptor (11). This concentration of complexes should, if in true equilibrium, depend on the total concentration of peptide in the membrane. Thus, we conclude that blocking is occurring at the APC cell surface, probably either at the site of the stimulation complex for the T cell receptor or by preventing a membrane-associated pool of peptide from associating with Ia.

While the features of this model are consistent with the antibody-blocking studies mentioned previously, several experimental considerations limited the structural conclusions that could be drawn directly from those studies alone. Cell surface blocking appeared to be clearly shown only in the study of Shevach et al.

(31); however, in that study the cells themselves were conjugated with TNP, and thus it was impossible to completely control for the effect of having TNP on other cell surface molecules. Localization of antibody-binding regions on antigen was not precisely known in any of the previous studies. Even in the study of Shevach et al. (31), although the defined hapten TNP was used, the sites of specific lysine modifications and their proximity to critical antigenic residues could not be known. The 35-Å-diameter Fab' portion of antibody (39) should have more access to recessed sites such as the Ia–lipid membrane junction as compared with the 55 × 55-Å binding face of avidin (40). However, the overall rotational sphere of an intact antibody molecule is much larger than that of avidin, and thus antibody can potentially sterically block moieties more remote from their binding site than can avidin. Though Fab' fragments in principle could minimize this latter concern, the affinity of univalent Fab' binding compared with bivalent binding has not permitted their successful use in studies of antibody blocking of presentation (31). The lower affinity of univalent binding of whole antibody to relatively dispersed antigen on the APC cell surface has been proposed as the reason for failure to see blocking in several optimized experimental systems (7, 31). Use of the biotin–avidin system with its K_d of 10^{-15} M (41) appears to overcome effectively such affinity limitations.

Thus, our data suggest that immunologically relevant antigen is at the cell surface, freely accessible to macromolecules the size of avidin.

Summary

Although studies of the association of antigen with APC have been complicated by antigen-processing requirements, recent studies have suggested that immunologically relevant antigen should be present on the APC surface. Nevertheless, blocking of antigen presentation with antibody to the antigen has not been demonstrable in most systems. To study this problem we developed a system using avidin to block presentation of amino-terminal biotinylated synthetic peptide 132–146 of sperm whale myoglobin (B132) to a murine T cell clone specific for this site in association with I-E^d. >95% specific inhibition was observed with doses of B132 equipotent to unmodified peptide. Specific blocking could be observed: (a) after pulsing APC with antigen, washing, and incubating for a chase period of 8–16 h before addition of avidin and T cells to assure adequate time for intracellular trafficking and maximal display of antigen on the cell surface, or (b) when monensin is present during the antigen pulse to inhibit such traffic. Therefore, the inhibition appeared to be occurring at the cell surface unless dissociation and reassociation were constantly occurring. To distinguish these, B10.GD APC (I-E^d-negative) were pulsed with antigen and cocultured with B10.D2 APC (I-E^d-positive). No detectable antigen presentation resulted. Thus, minimal dissociation and reassociation between antigen and APC occurs and, consequently, blocking by extracellular solution-phase binding of avidin to antigen is unlikely. Taken together, these data suggest that the blocking is occurring at the cell surface. Thus, under physiologic conditions, immunologically relevant antigen necessary for T cell activation appears to be present on the APC surface and is freely accessible to macromolecules the size of avidin.

These findings hold specific implications for models of antigen presentation for T cell recognition.

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