ANTIGEN RECOGNITION BY H-2-RESTRICTED T CELLS

I. Cell-free antigen processing*

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The role of the antigen-presenting cell in the generation of the T helper lymphocyte-mediated immune response has been the subject of intensive investigation. To date it has been well established that accessory cells take up, sequester in intracellular compartments, and re-express antigen at the cell surface membrane in an energy-requiring, dynamic process that requires cell viability (reviewed in references 1–3). Various investigators have shown that T cells recognize antigen in association with I-A and I-E molecules present on the surface of the accessory cell (4, 5) and that different antigen determinants may be recognized in association with distinct Ia epitopes (6–9).

There is considerable circumstantial evidence that for most soluble protein antigens the re-expressed surface antigen that is recognized by T cells is not in native form. Several studies have shown that T cells recognize chemically denatured forms of antigen just as well as native, globular protein (10, 11) and that small peptide antigen fragments can be as effective as the whole protein in generating T cell responses (12–14). Furthermore, kinetic studies have shown an approximately 1-h delay between initial antigen binding to accessory cells and effective antigen presentation to T cells (15–17). Agents that raise lysosomal pH, such as ammonia and chloroquine, inhibit the development of effective antigen presentation when given immediately after antigen binding to accessory cells but not at later times (18).

All of these findings have led to the concept of "antigen processing", which based on the information referred to above, has been interpreted to involve the obligatory partial degradation of internalized antigen by accessory cells followed by the re-expression on the surface membrane of antigenic fragments in a form that can be seen in association with Ia molecules by T cells.

There have been several obstacles in the way of defining antigen processing more precisely. First it has been impossible to distinguish which part of the antigen degradation seen in accessory cells is essential for antigen presentation and which part is not. Secondly, it has been difficult to determine whether or

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not antigen processing involves other modifications to the antigen in addition to degradation.

In this paper we report an experimental technique that has allowed us to make headway against both of these problems. We used the Ia^+ BALB/c B cell lymphoma, A20-2J, as an antigen-presenting accessory cell and the stimulation of interleukin-2 (IL-2)[1] production by a set of chicken ovalbumin (cOVA)-specific, I^d-restricted T cell hybridomas as the means of detecting immunologically relevant antigen. Under these conditions viable A20-2J presented native, denatured, and enzymatically or chemically derived peptides equally well to many hybridomas. Most critically however, glutaraldehyde-fixed A20-2J cells presented peptides, but neither native nor denatured intact antigen. We interpret these results to mean that for this antigen and this set of T cells partial antigen degradation is both a necessary and sufficient condition for antigen presentation.

Materials and Methods

Mice. The BALB/c By, B10.D2nSn, D2.GD, and D1.LP mice used to characterize the T hybridomas used in these experiments were originally purchased from the Jackson Laboratories, Bar Harbor, ME or were bred in our own facilities.

Antigens. Chicken (c) and turkey (t) ovalbumins (OVA) were purchased from the Sigma Chemical Co., St. Louis, MO. Red jungle fowl (jf) OVA, reeves pheasant (ph) OVA, and chiloe widgeon (w) OVA were prepared from eggs obtained courtesy of Ed Schmitt, curator, the Denver Zoo, Denver, CO by ammonium sulfate precipitation from egg white and carboxy-methyl agarose chromatography (19, 20). Bobwhite quail (q) OVA was prepared in a similar manner from eggs obtained from the GQF Manufacturing Co., Savannah, GA. Polyacrylamide gel electrophoresis showed that all these OVA preparations contained a major band that migrated with a molecular weight of ~43,000 daltons that co-migrated with the commercially obtained cOVA and tOVA.

Urea-denatured cOVA was prepared as previously described (11). After denaturation with 8 M urea, reduction with 0.2 M 2-mercaptoethanol, and alkylation with sodium iodoacetate, the preparation was sequentially dialyzed against distilled water, buffered saline, and finally balanced salts solution. Sephadex G-75 chromatography was used to separate the opalescent, partially aggregated denatured cOVA from contaminating low molecular weight fragments.

Enzyme digestions and chemical degradation of denatured chicken OVA were carried out as described (21). Trypsin digestion was performed by addition of 1% wt/wt trypsin (TPCK-treated, Worthington Biochemical Co., Freehold, NJ) to 200 mg protein in 0.1 M NH_4HCO_3, pH 8.2. After 4 h at 37°C, another identical aliquot of enzyme was added and the reaction was allowed to proceed overnight before lyophilization. Modification of lysine residues with citraconic anhydride (Eastman Kodak Co., Rochester, NY) before trypsin digestion of denatured chicken OVA (therefore allowing cleavage only at arginine residues) was performed as previously described (22). After trypsin digestion was complete, the lysine residues were unblocked by treatment with 0.04 M pyridine-acetate buffer, pH 3.5. Chymotrypsin digestion was effected by the addition of 2% alpha-chymotrypsin (Worthington Biochemical Co.) to 200 mg protein. After 4 h at 37°C the reaction was stopped by the addition of 160 μl 1 mM TPCK and the preparation lyophilized. For cyanogen bromide (CNBr) degradation, 168 mg protein was taken up in 70% formic acid and 1.68 g CNBr (Sigma Chemical Co.) added. Overnight incubation in the dark at room temperature was halted by lyophilization.

[1] *Abbreviations used in this paper:* c, chicken; cOVA, chicken ovalbumin; H-2, histocompatibility gene complex of the mouse; Ia, I-region associated antigens; I-A and I-E/C, the A and E/C subregions, respectively, of the I-region of the mouse H-2; IL-2, interleukin-2; jf, red jungle fowl; MHC, major histocompatibility complex; OVA, ovalbumin; ph, pheasant; q, bobwhite quail; SDS, sodium dodecyl sulfate; t, turkey, w, chiloe widgeon.

T Lymphocyte Hybridomas. The cloned, antigen-specific, H-2-restricted T lymphocyte hybridomas used in this study were produced and characterized in a similar manner to that described previously (23). Briefly, 6–8-wk old normal BALB/c By mice were injected with 100 μg cOVA in complete Freund's adjuvant at the base of the tail. 7 d later the draining lymph nodes were excised and the cells cultured for 5 d in the presence of antigen in modified Clicks medium. After a subsequent 3-d expansion in IL-2-containing medium, the antigen-reactive T cell blasts were fused to an azaguanine-resistant variant of the AKR thymoma BW5147 with polyethylene glycol. Resultant hybrids were selected for in hypoxanthine/aminopterin/thymidine-containing media and were screened for the production of IL-2 when cultured with cOVA in the presence of A20-2J B lymphoma cells. A subset of these initial hybridomas were selected for cloning based on their unique patterns of cross-reactivity with a series of heterologous OVA's and their alloreactivity against cells bearing H-2 types other than *d*. Cloning was performed at limiting dilution under conditions in which the selected clones had less than a 2.4% chance of arising from more than one cell. In general, one clone of each hybrid was selected for use and its I subregion restriction determined by mapping antigen responsiveness on BALB/c By ($I\text{-}A^d$, $I\text{-}E^d$), B10.D2 ($I\text{-}A^d$, $I\text{-}E^d$), D2.GD ($I\text{-}A^d$,$I\text{-}E^{absent}$), and D1.LP ($I\text{-}A^b$,$I\text{-}E^{absent}$) irradiated spleen cells. This MHC-restriction was confirmed by assaying inhibition of antigen presentation by the $I\text{-}A^d$, $I\text{-}E^d$-expressing A20-2J cells using the anti-$I\text{-}A^d$ (but not $I\text{-}E^d$)-specific MK-D6 and the anti-$I\text{-}A^d$, $I\text{-}E^d$-specific M5/114 monoclonal antibodies. In addition the alloreactivity of each hybrid was tested with spleen cells from a set of H-2 congenic and recombinant mice covering the k, b, s, q, and f haplotypes. The antigenic fine specificities, I subregion restrictions, and alloreactivities of the T hybridomas are shown in Table I. In addition to the antigens displayed in Table I these T hybridomas were negative for IL-2 production when assayed for reactivity on A20-2J presenting cells to duck OVA, goose OVA, chicken conalbumin, purified protein derivative of tuberculin, keyhole limpet hemocyanin, human gamma globulin, and the synthetic polymer poly-L-(Tyr,Glu)-poly D,L-Ala--poly L-Lys (data not shown). Also, with the exception of DO-11.10, none of these hybridomas reacted to cOVA or any of the other OVA's when presented by presenting cells of other than $H\text{-}2^d$ type.

Antigen-presenting Cells. The $I\text{-}A^d$, $I\text{-}E^d$-positive A20-2J (known previously as L10A.2J) B lymphoma line that has previously been shown to present antigen in an MHC-restricted fashion to T cells (24) was used in these experiments. For use in antigen presentation, A20-2J cells were centrifuged from bulk culture and resuspended to appropriate concentrations for addition to micro-culture wells. Antigen-pulsed A20-2J were cultured in the presence of 1 mg/ml OVA for 2 h at 37°C, washed twice with balanced salts solution, and added to micro-culture wells. Our studies with cOVA and previous studies with other antigens (25, 26) showed that at least 60 min was required for processing of protein antigens by A20-2J. For fixation, cells were washed twice with balanced salts solution (after antigen pulsing, if desired) and 5×10^6 cells per ml in balanced salts solution were fixed by the addition of glutaraldehyde (Sigma Chemical Co.) to a final concentration of 0.05%. After 30 s at room temperature the fixation was stopped by the addition of an equal volume of 0.2 M lysine (Sigma Chemical Co.) in balanced salts solution, pH 7.4. The cells were then centrifuged and washed once before use. Cells fixed under these conditions could be stored at 4°C for up to 3 wk without impairing their antigen-presenting capabilities.

Antigen Stimulation Cultures and Assay for IL-2 Production. Assay for antigen-specific stimulation of IL-2 production by T hybridomas was performed as previously described (23) in 96-well tissue culture-treated micro-culture plates (Flow Laboratories, McLean, VA) using 1×10^5 A20-2J B lymphoma presenting cells, 1×10^5 T hybridoma cells and (when desired) 1 mg/ml antigen in 0.3 ml complete medium. Cultures were incubated for 24 h at 37°C whereupon the presence of IL-2 in the culture supernates was assayed by using the IL-2-dependent HT-2 cell line. IL-2 titers are expressed in U/ml.

Amino Acid Incorporation by Glutaraldehyde-fixed A20-2J Cells. Indicated number of A20-2J, freshly 0.05% glutaraldehyde-fixed A20-2J, and A20-2J cells that had been fixed 5 d previously with 0.05% glutaraldehyde then stored at 4°C, were pulsed for 8 h at 37°C in

0.2 ml of medium containing [^{3}H]leucine (600 μM, 33 μCi/μmole, New England Nuclear, Boston, MA). Cultures were then harvested onto glass fiber paper, washed, fixed with 10% trichloroacetic acid and absolute methanol and [^{3}H]leucine incorporation enumerated on a Beckman LS-2800 scintillation counter.

Catabolism of ^{125}I-Labeled cOVA. Denatured cOVA was labeled with ^{125}I by the Chloramine T method (26). In order to assess the ability of A20-2J cells to catabolize denatured cOVA after fixation, 5×10^5 fixed or unfixed A20-2J cells were incubated for 24 h at 37°C in 1.0 ml of medium containing 1 mg ^{125}I-cOVA (~ 2 μCi/mg). The cells were then collected and washed once with balanced salt solution. The ^{125}I-cOVA remaining associated with the cells was examined by dissolving the cell pellet in 1.5% sodium dodecyl sulfate (SDS) containing 2-mercaptoethanol and analyzing a portion of the lysate using SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (27). A 12.5% gel was used. The ^{125}I-labeled peptides were visualized using Kodak XAR-S film exposed at −70°C.

Results

Antigenic Fine Specificities of the T Hybridomas. The T cell hybridomas used in these experiments were prepared from three separate hybridizations of BALB/c anti-OVA T cell blasts to BW5147 thymoma cells. T hybridomas prepared under identical conditions have already been characterized (23) and, therefore, the general properties of these cells will not be discussed. As can be seen in Table I, when assayed by their ability to be stimulated to produce IL-2, each of the selected cOVA-specific hybrids had a unique specificity defined by the combination of its I-region restriction specificity, its cross-reactivity pattern with a set of heterologous OVA's, and its pattern of alloreactivity on non-*H-2*^{d} presenting cells. In constructing this set of cOVA-specific hybridomas, each with a unique receptor, we hoped to cover as many of the various ways as possible in which BALB/c T cells could recognize cOVA in association with either the I-A^d or I-E^d molecule. In retrospect, based on the evidence presented below, it would appear that this set of hybridomas define a number of different antigenic regions on the cOVA molecule, although some members of the set appear to recognize

TABLE I

Cloned BALB/c-derived, cOVA-specific, I^d-restricted T Cell Hybridomas Used in These Studies

Hybridoma	IL-Production (U/ml) in response to A20-2J cells presenting these antigens							I-sub region restriction	Alloreactivity	
	cOVA	jfOVA	phOVA	tOVA	qOVA	wOVA	No antigen		IL-2	Specificity
									U/ml	
3DO-26.1	640	160	320	320	—	—	—	I-E^d	—	none
3DO-36.6.1	80	320	—	640	80	—	—	I-E^d	—	none
4DO-63.10	320	—	640	320	—	—	—	I-E^d	—	none
3DO-54.8	640	320	—	—	160	—	—	I-A^d	80	I-A^{f,s}
									10	I-A^k
3DO-54.6	640	160	—	—	—	—	—	I-A^d	—	none
DO-11.10	640	320	—	—	—	—	—	I-A$^{d\,or\,b}$	20	I-A^b
3DO-18.3	640	640	320	—	320	—	—	I-A^d	—	none
3DO-46.4	320	160	320	—	—	—	—	I-A^d	—	none
3DO-20.10	640	—	—	—	—	—	—	I-A^d	—	none
4DO-11.7	640	—	—	640	—	320	—	I-A^d	—	none

Antigen stimulation cultures and IL-2 assay performed as described in the Materials and Methods. Data presented is averaged from numerous assays over a several week period. Variance of IL-2 responses are ± one dilution except for negative responses which were never positive.

the same general region of cOVA, with fine specificity differences defined by their patterns of cross-reactivity.

There are several points worth noting about the reactivity patterns of the hybridomas. 3DO-36.6.1, although produced from cOVA-immune animals, consistently responded poorly to cOVA but strongly to tOVA. 3DO-54.6 and 3DO-54.8 are clones that were derived from the same primary hybridoma well, although they clearly had different fine specificity patterns. It is possible that these were two independent hybrids which happened to grow in the same well, or that these were derived from two progeny of the same hybrid that differ in specificity because of a receptor mutation or differ in antigen sensitivity because of differences in receptor density, for example. We cannot distinguish among these possibilities at present, so both hybrids were included in the study. DO-11.10 had a very unusual specificity in that its very weak response to I-A^b as an alloantigen was dramatically improved by the addition of cOVA.

Effect of Fixation on Antigen Presentation. In previous studies using a number of I-region restricted T cell hybridomas specific for protein antigens, we and others (17, 26) reported evidence for the importance of antigen processing by showing that antigen-presenting cells preincubated for several hours with antigen could be fixed without impairing their ability to present antigen. On the other hand, antigen added after fixation was not presented. Table II shows that cOVA presentation by A20-2J to a number of the hybrids listed in Table I followed similar rules. These cOVA-specific hybrids responded to cOVA-pulsed A20-2J cells, although not as well as to cOVA continuously present at high levels with A20-2J cells. Glutaraldehyde fixation of the previously pulsed A20-2J cells had no effect on their ability to present cOVA. Most importantly, A20-2J cells that were fixed without pulsing with cOVA were unable to present cOVA even when tested with high concentrations of continuously present cOVA.

These findings reconfirmed the reports by us and others supporting the conclusion that protein antigens such as cOVA are not recognized in a native form by I-region restricted T cells. Rather they suggest that the antigen-

TABLE II

Response of T Hybridomas to Antigen-pulsed and 0.05% Glutaraldehyde-fixed A20-2J Presenting Cells

Hybridoma	U/ml IL-2 production in response to:			
	A20-2J plus 1 mg/ml added cOVA	cOVA-pulsed A20-2J	Glutaraldehyde-fixed cOVA pulsed A20-2J	Glutaraldehyde-pre-fixed A20-2J + 1 mg/ml added cOVA
3DO-18.3	1,280	160	160	0
3DO-54.8	1,280	160	160	0
3DO-54.6	640	20	20	0
DO-11.10	640	80	80	0
4DO-11.7	1,280	40	40	0

Conditions of assay, antigen pulsing and glutaraldehyde fixation of presenter cells as described in the Materials and Methods. Data presented is averaged from numerous assays. Variance of IL-2 responses are ± one twofold dilution except for negative responses, which were never positive.

presenting cells must alter or process the antigen to a form that can be recognized.

Ability of Glutaraldehyde-fixed A20-2J Cells to Present Enzymatically and Chemically Generated OVA Peptides to the T Hybridomas. We then designed a set of experiments to attempt to define more precisely what the essential antigen processing steps might be. Some of the possibilities we wished to distinguish were (*a*) that processing simply involved the denaturation of the protein, (*b*) that processing required the proteolytic cleavage of the protein into smaller peptides, and (*c*) that processing required other intracellular modifications to the protein, e.g. covalent coupling to membrane components during membrane biogenesis.

To test these possibilities we examined the ability of A20-2J cells before and after fixation to present exogenously added native cOVA, denatured cOVA, and cOVA peptides prepared by either enzymatic or CNBr cleavage of denatured cOVA. We reasoned that if denaturation was the essential feature of processing then this inability of fixed A20-2J cells to present native cOVA should be overcome by denaturation of the antigen. Similarly, if partial degradation was the critical step in processing then at least for some hybrids we should be able to restore the presenting ability of A20-2J cells by the right set of cleavages of the cOVA molecule in vitro. Finally, if some other essential intracellular event was required for processing then none of our extracellular manipulations should restore the presenting ability of the fixed A20-2J cells.

The results of these experiments are summarized in Table III. The left-hand side of Table III shows the production of IL-2 by the T hybridomas in response to cOVA, denatured cOVA, the tryptic and chymotrypic peptides, the tryptic peptides of citraconylated cOVA, and the cyanogen bromide-generated peptides of cOVA as presented by nonfixed A20-2J cells. As predicted by previous experience with protein antigens, all but two of the hybrids responded to both the native and denatured form of cOVA indicating that in general the T cells do not recognize conformational determinants found in the native molecule. Two hybrids failed to respond to denatured cOVA. In the case of 3DO-36.6.1, as discussed below, this failure to respond appeared to be related to antigen processing rather than to the destruction of the recognized determinant by denaturation. On the other hand, the hybridoma 4DO-11.7 was apparently reactive to a determinant on native cOVA that was destroyed by the urea denaturation or by the alkylation of cysteine residues.

Also, most of the hybridomas could be shown to react with at least one of the peptide preparations to a similar extent as to the native or denatured intact molecule, although, as might be expected, different hybridomas showed different patterns of reactivity to cOVA fragmented by each of the methods.

The right half of Table III shows the results of testing the ability of previously fixed A20-2J cells to present the various cOVA preparations. As shown in Table II, these fixed cells could not present native cOVA to any of the hybridomas demonstrating the need for some active, metabolic processing event before presentation. Denaturation of the cOVA did not restore its ability to be presented by the fixed A20-2J cells, indicating that denaturation alone did not account for processing. Remarkably, in 7 out of 10 hybridomas, the antigen-presenting ability of the fixed cells was restored by at least one of the cOVA fragmentation methods.

TABLE III

Response of T Hybridomas to Denatured cOVA and Its Peptides on Unfixed and 0.05% Glutaraldehyde-fixed A20-2J Presenter Cells

Hybridoma	U/ml IL-2 production in response to: A20-2J plus 1 mg/ml cOVA						Fixed A20-2J plus 1 mg/ml cOVA					
	Native	Denatured	Trypsin digested denatured	Citraconylated trypsin digested denatured	Chymotrypsin digested denatured	CNBr degraded denatured	Native	Denatured	Trypsin digested denatured	Citraconylated trypsin digested denatured	Chymotrypsin digested denatured	CNBr degraded denatured
3DO-26.1	320	320	10	320	0	320	0	0	0	0	0	40
3DO-36.6.1	20	0	0	320	0	0	0	0	0	0	0	0
4DO-63.10	160	160	20	320	0	320	0	0	0	10	0	80
3DO-54.8	1,280	1,280	640	640	80	640	0	0	320	640	20	320
3DO-54.6	320	80	320	640	0	320	0	0	160	160	0	40
DO-11.10	640	640	640	640	320	640	0	0	640	640	160	320
3DO-18.3	1,280	1,280	0	0	0	1,280	0	0	0	0	0	640
3DO-46.4	160	160	0	0	0	40	0	0	0	0	0	20
3DO-20.10	320	320	640	640	0	0	0	0	160	40	0	0
4DO-11.7	1,280	0	0	0	0	0	0	0	0	0	0	0

Conditions of assay, glutaraldehyde fixation of presenter cells, and antigen preparations described in the Materials and Methods. Data presented is averaged from several separate assays. Variance of IL-2 responses are ± one dilution in the assay for IL-2 except for negative responses which were never positive.

The presence of both antigen (cOVA peptides) and fixed A20-2J presenters are required for recognition. The anti-I subregion-specific MK-D6 (anti-I-A^d) and M5/114 (anti-I-A, E^d) monoclonal antibodies inhibit these responses in the same manner as with the intact antigens presented by viable A20-2J cells (data not shown). We consider this evidence that the OVA-specific H-2-restricted T hybridomas are recognizing the OVA peptides on the fixed presenters in an identical fashion as they recognize native antigen presented by viable A20-2J or macrophage presenters. Indeed, when the T hybridomas 3DO-18.3 and 3DO-54.8 were assayed for specific secretion of IL-2 in response to cOVA peptides on 0.1% glutaraldehyde-fixed BALB/c resident peritoneal cells, the same pattern of reactivity was observed as that seen with fixed A20-2J presenters (data not shown). The phenomenon is not unique, therefore, to B cell or tumor cell presenters as it can be demonstrated with fixed normal macrophages.

These results lead us to the major conclusion of this study: that for this set of cOVA-specific T cell hybridomas, the fragmentation of the cOVA to peptides is *both* necessary and, more importantly, sufficient to explain antigen processing. These results argue strongly against an essential role in processing for intracellular modifications other than antigen degradation.

There are a number of other important observations to be made from the data in Table III. Hybridoma 4DO-11.7 failed to respond to denatured cOVA or any of the cOVA peptide preparations whether presented by fixed or nonfixed A20-2J. As mentioned above, the most straightforward interpretation of this result is that the denaturation of cOVA permanently destroyed the determinant recognized by the hybridoma, either because the determinant is conformational or fails to survive the alkylation of the cOVA sulfhydryl groups.

The response pattern of 3DO-36.6.1 was particularly unexpected. As shown in Table I, although this hybridoma was derived from a cOVA-immunized mouse, it showed a poor response to cOVA, but a strong response to tOVA. This might have indicated a determinant in cOVA with very low affinity for the receptor on 3DO-36.6.1 that was dramatically improved by a species-specific amino acid substitution found in tOVA. The data in Table III, however, suggests an alternate explanation. The fact that trypsin treatment of citraconylated cOVA restored a strong response by 3DO-36.6.1 to this antigen indicates the presence of an equally effective determinant in both cOVA and tOVA. It would appear that this determinant can be processed efficiently by A20-2J from tOVA but not from cOVA unless helped by the appropriate extracellular enzyme treatment.

In no case did we observe a response with fixed A20-2J cells that was not also evident with unfixed cells. However, there were several examples where cleavage with a particular method yielded cOVA peptides that were presented by nonfixed but not by fixed A20-2J cells. This was particularly true with CNBr cleavage or digestion of the citraconylated cOVA with trypsin. Since these methods could be predicted to yield the largest cOVA fragments, it is possible that in some cases the degradation of the cOVA was not sufficient to release the relevant peptide. In this respect it will be interesting in the future to establish whether combining these or other fragmentation methods might reveal the relevant peptide in these partially degraded proteins.

While the data presented in Tables I and III support the conclusion that the

hybrids used in these studies each had a unique receptor defined by its fine specificity, the patterns of reactivity seen in Table III suggest that certain of these hybrids nevertheless may be responding to the same portion of the cOVA molecule. For example, the responses of 3DO-26.1 and 4DO-63.10 to the cOVA peptides were strikingly similar, suggesting a particular portion of the cOVA molecule may be preferentially seen in association with I-E^d. Similarly 3DO-54.6, 3DO-54.8, and DO-11.10 had similar reactivity patterns with the cOVA fragments, again suggesting a response to a common region of the cOVA molecule. In preliminary experiments fractionating the cOVA tryptic peptides, we have strengthened this conclusion with the finding that each of these three hybrids apparently recognize the same tryptic peptide.

Evidence for Fixation of A20-2J. It was critical to the interpretation of our results that the fixation of A20-2J cells be effective. Others have shown that treatment of different cell lines with varying amounts of glutaraldehyde can eliminate amino acid or nucleic acid incorporation and antibody plus complement or cytotoxic T cell recognition and lysis (28). Although the data presented here is for 0.05% glutaraldehyde-fixed A20-2J cells, presentation of cOVA digests occurs in an undiminished fashion, as evidenced by T cell secretion of IL-2, by A20-2J cells prefixed with concentrations of glutaraldehyde up to 0.3% for 30 s at room temperature (data not shown). The fact that the glutaraldehyde fixation totally eliminated the ability of A20-2J to present nondegraded cOVA certainly indicated a severe diminution of the cells' metabolic capabilities. However, we performed several additional control experiments to assess further the metabolic activity of these cells. We determined that these fixed cells were unable to proliferate in culture (data not shown). Furthermore, as shown in Table IV, the fixation procedure reduced protein synthesis as evidenced by the ability of A20-2J to incorporate [^{3}H]leucine into acid insoluble material, to 1% or less of that seen in unfixed cells. We haven't determined as yet whether this small residual activity reflects actual protein synthesis or simply trapping of a small amount of the free [^{3}H]leucine in the acid-precipitated cells. In addition, the stability of the fixed cells was tested. Fixed A20-2J cells that had been stored at 4°C for up to 3 wk were unaffected in their ability to present cOVA peptide fragments to the cOVA-specific hybrids (Table V). We assessed the ability of fixed or unfixed A20-2J cells to degrade denatured cOVA. Cells were incubated overnight with

TABLE IV

Inhibition of Protein Synthesis by 0.05% Glutaraldehyde Fixation

No. of A20-2J cells	[^{3}H]Leucine incorporated into TCA precipitable material (cpm × 10^{-3})		
	Nonfixed A20-2J	Freshly glutaraldehyde-fixed A20-2J	5-d previously glutaraldehyde-fixed A20-2J
5×10^5	101	1.4	0.4
1×10^5	20.7	0.1	0.1

Assay conditions described in the Materials and Methods. Data shown are averaged from duplicate counts of a single experiment from which background counts have been subtracted.

TABLE V

Stability of 0.05% Glutaraldehyde-fixed A20-2J Cells

Hybrid	IL-2 production (U/ml) in response to:						
	Non-fixed A20-2J plus	Freshly fixed A20-2J plus			A20-2J fixed 3 wk earlier plus		
	native cOVA	native cOVA	trypsin treated-cOVA	CNBr treated-cOVA	native cOVA	trypsin treated-cOVA	CNBr treated-cOVA
3DO-54.8	640	0	640	nt	0	320	nt
3DO-18.3	640	0	nt	640	0	nt	320

Conditions of assay and glutaraldehyde fixation of presenter cells as described in the Materials and Methods. Data presented is averaged from numerous assays. Variance of IL-2 responses are ± one twofold dilution except for negative responses which were never positive.
nt, not tested.

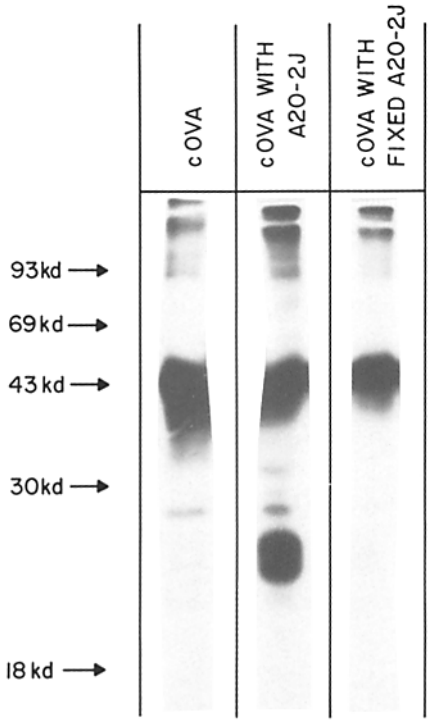

FIGURE 1. Failure of fixed A20-2J cells to catabolize cOVA. Fixed and unfixed A20-2J cells were incubated for 24 h with ^{125}I-labeled denatured cOVA and the cell-associated radiolabeled peptides analyzed by SDS-PAGE as described in the Materials and Methods. Lane *1*, intact ^{125}I-cOVA (250,000 cpm). Lane *2*, ^{125}I-cOVA associated with unfixed A20-2J (100,000 cpm). Lane *3*, ^{125}I-cOVA associated with fixed A20-2J (40,000 cpm).

^{125}I-labeled denatured cOVA and then washed to remove unbound antigen. Of the input radioactivity, ~17% remained associated with the unfixed A20-2J cells, and 4% with the fixed A20-2J cells. The cell pellets were dissolved in SDS and the lysates and intact ^{125}I-denatured cOVA were analyzed by SDS-PAGE

(Fig. 1). A considerable portion of the cOVA associated with the unfixed A20-2J cells was degraded to ^{125}I-labeled peptides ranging in molecular weight from ~20 to 35 kdaltons. There was no evidence of degradation of the cOVA associated with the fixed A20-2J cells.

Taken together these results strengthen our overall conclusion that metabolically inert fixed cells cannot process antigen but are very effective at presenting preprocessed antigenic peptides.

Discussion

Recognition of protein antigens in vitro by I-region restricted, antigen-specific T cells has been known for several years to require presentation of the antigen on an Ia-bearing cell. Although originally macrophages were thought to be the primary presenting cell (29, 30), there now exists convincing evidence that other cell types, especially B cells, can take up antigen effectively and present it in an I-region restricted fashion (24, 31).

Observations, such as the lag time between antigen uptake and antigen presentation (15, 16), the equal effectiveness of native, denatured, and fragmented antigen in stimulating secondary T cell responses (10–14), and the inhibitory effect of fixatives given before but not after incubation of the presenting cell with antigen (17, 26) have all led to the concept of antigen "processing." This term has commonly been used to signify the uptake, internalization, partial degradation and/or modification, and re-expression on the cell surface of fragments of protein antigens that can be seen in association with I-region molecules by T cells.

Although the circumstantial evidence in favor of antigen processing is strong, there is little direct evidence for any of the steps involved. A major obstacle in attacking this problem experimentally has been the inability to follow or control the processing of antigen in viable presenting cells. Thus, one is unable to distinguish which of the catabolic pathways followed by the antigen are essential to antigen presentation and which are not.

The experimental system that we developed to study this problem was the stimulation of IL-2 production from a set of cOVA-specific, I^d-restricted T cell hybridomas by cOVA presented by the Ia^+, BALB/c B cell lymphoma, A20-2J. This system had a number of advantages. First, all of our previous experience with these T cell hybridomas indicated that their response depended only on the recognition of cOVA plus I^d molecules with no requirement for mediators such as, interleukin-1, from the presenting cell. Thus we felt we could isolate the events necessary for antigen presentation from other events related to T cell activation. Second, our set of T cells were clonal and selected to cover as many of the various determinants on cOVA as possible. We expected that the individual determinants of the OVA molecule may be processed in different ways; thus, the complexity of antigen processing observed with a heterogenous set of responding T cells might become simpler at the clonal level. Third, we had shown that B cell lymphomas, such as A20-2J, were very efficient at antigen presentation in that when compared to normal macrophages these cells took up less antigen and degraded antigen less, but were equally or more effective in antigen presentation. Thus, we felt that distinguishing essential from nonessential

antigen degradation might be easier in these cells. Finally, we took advantage of our recent observation that the antigen-presenting abilities of A20-2J and other types of presenting cells were not destroyed by fixation, provided that the cells were allowed sufficient time to process the antigen before fixation. We reasoned that the events essential to antigen processing might be definable outside the cell by using these presenting cells whose own metabolic capabilities were neutralized by fixation.

The essential finding of our studies presented here was that A20-2J cells fixed with glutaraldehyde were unable to present cOVA unless the molecule had been previously fragmented either chemically or enzymatically. As expected, the different fragmentation methods were more or less effective depending on the responding T cell hybridoma clone, indicating the recognition of particular regions of the OVA molecule by individual T cell hybridomas. These results offer direct evidence that antigen fragmentation is generally a required step in processing of the soluble protein antigen such as cOVA.

Our results also led us to the unexpected conclusion that for this experimental system, antigen fragmentation was sufficient to define antigen processing since once degraded to peptides the antigen could be presented by apparently metabolically inert Ia^+ cells. The strength of this conclusion was dependent on the effectiveness of the glutaraldehyde fixation of A20-2J. Our experiments demonstrated that these fixed cells were (*a*) not viable, (*b*) unable to functionally process or biochemically degrade cOVA, (*c*) unable to synthesize protein, and (*d*) stable in their presenting function upon long term storage. These results certainly place limitations on what further modifications the fixed A20-2J cells might make on the cOVA peptide fragments. Nevertheless, the fixation conditions used in our experiments were quite mild and we cannot as yet rule out formally the possibility that some cellular functions (e.g. surface membrane enzymes) survived the fixation and storage to play an essential role in the further modification of the antigen peptides.

In summary, the data presented here confirms the central role of antigen degradation in antigen processing for presentation to T cells. This experimental system offers great promise in both investigating the essential steps in antigen processing and defining the nature of the antigen as it is recognized in association with I-region molecules by T cells.

Summary

We examined the ability of a set of cloned chicken ovalbumin (cOVA)-specific, I^d-restricted, T cell hybridomas to produce interleukin-2 in response to cOVA presented by the Ia^+ B cell lymphoma line, A20-2J. Although viable A20-2J cells presented native, denatured, and fragmented cOVA more or less equally well, A20-2J cells that were glutaraldehyde-fixed could present only enzymatically or chemically fragmented cOVA. These results suggest that antigen fragmentation may be both necessary and sufficient to define accessory cell processing of soluble antigens so that they may be recognized in association with I-region molecules by T cells.

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