

Epitope-directed Processing of Specific Antigen by B Lymphocytes

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Abstract. Proteolytic processing of specific antigen was studied using Epstein Barr virus transformed B-lymphoblastoid cells expressing membrane IgG against tetanus toxin. As previously reported (Watts, C., and H. W. Davidson. 1988. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1937-1945), receptor-mediated endocytosis of monovalent antigen bound at 0°C began immediately upon shifting the cells to 37°C. In contrast, degradation of antigen, assessed either by the release of acid-soluble radiolabel into the incubation medium, or by SDS-PAGE analysis of total cell-associated antigen, proceeded after a lag of 10-20 min. Degradation was abolished by exposure of the cells to metabolic inhibitors, or by incubation at 20°C, and inhibited in a dose-dependent fashion by chloroquine and by the lysosomal protease inhibitors leupeptin, E-64, and pepstatin A.

Analysis of the cell-associated radiolabel by SDS-PAGE and autoradiography after incubations at 37°C revealed the time-dependent generation of distinct antigen fragments. Virtually quantitative immunoprecipitation of these fragments was obtained using a monoclonal anti-human IgG antibody, indicating that the antigen/mIg complex is the initial substrate for processing. We show that the pattern of fragmentation observed varies from one B cell line to another (a) depending on the epitope through which the antigen is bound and endocytosed and (b) depending on whether additional epitopes in the antigen are complexed with anti-tetanus Fabs. The implications of these results for the presentation of major histocompatibility complex restricted antigen fragments, and for intracellular trafficking of ligand/receptor complexes are discussed.

THE generation of an immune response involves the collaboration of several different cell types. Thus for the production of an antibody both B and T lymphocytes specific for the particular antigen are required; each cell expressing a surface receptor capable of recognizing a distinct region within the molecule. However, whereas the B cell epitope is generally present in the native antigen, T cells only recognize the antigen when it is associated with the major histocompatibility complex (MHC)¹ glycoproteins, a restriction which dictates that the antigen must be processed and presented by an appropriate antigen presenting cell (APC) (for reviews see Unanue, 1984; Schwartz, 1985; Allen, 1987; Lanzavecchia, 1988).

Recent studies have suggested that there are distinct routes for the association of antigen fragments with class I and class II MHC molecules. The former appears to involve processing of the antigen in a chloroquine-insensitive (nonendosomal) compartment, and is normally dependent upon expression of the antigen by the cell involved (e.g., after viral infection; Townsend et al., 1985; Morrison et al., 1986), although this requirement can be by-passed by delivery of exogenous antigen to the cytosolic compartment (Moore et al., 1988). In contrast, association with class II MHC glycoproteins generally occurs after processing in a chloroquine-

sensitive compartment after exposure of the APC to exogenous antigen (Chesnut et al., 1982; Ziegler and Unanue, 1982; Morrison et al., 1986), although chloroquine-sensitive association of endogenous antigen with Class II molecules has also been observed (Jin et al., 1988).

The situation is still more complex since the expressed T cell repertoire to many proteins appears to be focused upon a limited number of immunodominant regions (Benjamin et al., 1984). The particular T cell epitopes used are determined not only by the ability of a particular peptide to bind to the MHC molecules expressed by the APC, but also by the molecular context in which it is located. Thus molecular features distinct from the T cell determinant itself can directly influence the efficiency of its presentation, perhaps by rendering the part of the molecule in which it is located more or less susceptible to proteolysis, or by favoring the production of other fragments which can compete for the MHC binding site (Gammon et al., 1987; Brett et al., 1988; Adorini et al., 1988). Clearly the antigen processing step is critical in determining the nature of the immune response generated.

In vitro, antigen processing can often be mimicked by limited proteolysis, with the generation of fragments capable of binding to MHC glycoproteins either on the surface of cells (Shimonkevitz et al., 1983; Townsend et al., 1986) or in isolated planar liposomes (Watts et al., 1984). Such studies have defined minimal peptides (typically 7-17 amino acids in length) within a variety of well-characterized anti-

1. *Abbreviations used in this paper:* APC, antigen presenting cell; MHC, major histocompatibility complex; mIg, membrane immunoglobulin.

gens which are capable of stimulating antigen-specific T cells, thereby allowing critical residues involved in the recognition of such peptides by each receptor to be identified (Guillet et al., 1986; Allen et al., 1987; Buus et al., 1987; Rothbard and Taylor, 1988). However to date the molecular events which occur at the surface of, and within, the APC remain largely obscure; no one has yet succeeded in isolating or identifying a physiological antigen fragment (Allen, 1987).

Most previous investigations of the cellular fate of exogenous antigens have been conducted using macrophages as APCs. These have indicated that the antigen must first be endocytosed, and is then "processed" in an "acidic" (chloroquine sensitive) compartment before reexpression on the cell surface in association with class II MHC (Unanue, 1984; Schwartz, 1985; Allen, 1987). Several studies have also shown a direct correlation between the release of acid soluble antigen fragments by APCs and their ability to stimulate relevant T cells (e.g., Ziegler and Unanue, 1982), and that presentation can be inhibited by exposure of the APC to inhibitors of "lysosomal" proteases such as leupeptin (Streicher et al., 1984; Buus and Werdelin, 1986). Moreover, a recent report demonstrated that the MHC class II restricted presentation of an epitope of pigeon cytochrome *c* was prevented when both the antigen and MEP (a cathepsin L precursor) were simultaneously endocytosed, apparently after maturation of the protease and subsequent destruction of the T cell epitope (McCoy et al., 1988). Thus a role for the endosome/lysosome pathway in antigen processing is generally accepted, although the precise details remain obscure.

Besides macrophages, several other lymphoid cells which constitutively express class II MHC can function as APCs, including B lymphocytes. However, in contrast to other APCs, individual B cells express receptors (membrane immunoglobulins [mIg]) specific to particular antigens and are consequently considerably more efficient at presenting their specific antigen (Chesnut and Grey, 1981; Rock et al., 1984; Lanzavecchia, 1985). However, to date few studies on the molecular events which occur during antigen processing by B cells have been carried out, mainly due to the difficulty in obtaining antigen-specific cells.

Recently several tetanus toxin/toxoid specific B cell clones have been isolated, and have been shown to endocytose monovalent antigen (Lanzavecchia, 1985; Watts and Davidson, 1988). We now report that after endocytosis antigen is degraded in a time- and temperature-dependent manner and, that clones recognizing distinct epitopes within tetanus toxin generate epitope-dependent antigen fragments demonstrating a direct influence of the receptor on antigen processing by B cells.

Materials and Methods

Materials

Tissue culture reagents were supplied by Flow Laboratories (Irvine, Ayrshire, Scotland) and electrophoresis reagents by BDH Chemicals Ltd., Poole, England. Monoclonal anti-human IgG antibodies QE11 and 312H were obtained from The Binding Site Ltd. (Birmingham, UK) and affinity absorbed rabbit anti-mouse Ig from Dako Ltd. (High Wycombe, UK). The latter was coupled to CNBr activated Sepharose (Pharmacia Fine Chemicals, Milton Keynes, UK) at ~5 mg/ml swollen resin by procedures recommended by the suppliers. Partially purified tetanus toxin and toxoid were

kindly supplied by Dr. N. Fairweather and Mr. A. Sheppard (Wellcome Biotech). Monovalent antigen, Fab fragments of monoclonal anti-tetanus toxin antibodies, and radio-iodinated proteins were prepared as described previously (Watts and Davidson, 1988). All other chemicals were obtained from Sigma Chemical Co. (Poole, UK) unless otherwise specified.

Cell Culture

Epstein Barr virus-transformed B cell lines 8.5, 11.3 (donor K.K.), and A46 (donor A.L.) were kindly supplied by Dr. A. Lanzavecchia (Basel Institute of Immunology) and maintained in RPMI 1640 supplemented with 10% (vol/vol) heat denatured fetal calf serum, 2 mM glutamine, 1 mM Na pyruvate, 50 µg/ml streptomycin, 50 IU/ml penicillin, and nonessential amino acids in a 5% CO₂/95% air atmosphere.

Binding and Endocytosis of Antigen

B cells were removed from culture, collected by centrifugation (4 min at 500 g), and washed in ice-cold PBS containing 5 mg/ml BSA. Washed cells were resuspended in PBS/BSA at a density of 2×10^7 /ml and incubated at 0°C in the presence of 2 µg/ml ¹²⁵I toxin for 2 h with occasional mixing. The cells were then collected by centrifugation, and washed extensively with PBS/BSA to remove unbound antigen. Specific binding, as assessed by duplicate incubations conducted in the presence of a 100-fold excess of unlabeled tetanus toxoid, was >90%.

In general, labeled cells were resuspended in growth medium at a density of 4×10^6 /ml and incubated in a CO₂ incubator at 37°C for times as indicated. Aliquots of the chase incubation media were retained and acid soluble radiolabel determined after precipitation with TCA (final concentration 10% [wt/vol]). To assess the proportion of cell-associated antigen remaining on the cell surface, in some experiments the cells were resuspended in PBS (5×10^6 cells/ml) containing 1.5 mg/ml pronase (Sigma protease XIV) and 100 µg/ml DNase II, and incubated on ice for 1 h.

SDS-PAGE Analysis

Cell pellets were resuspended in 50 mM Tris-HCl, pH 6.8, containing 12% (vol/vol) glycerol, 4% (wt/vol) SDS, and 0.01% Coomassie blue G (SDS sample buffer), and homogenized by repeated passage through a 25-gauge needle. After incubation at 40°C for 30 min, and centrifugation at 13,000 g for 10 min, the samples were loaded onto polyacrylamide slab gels prepared according to the method of Schägger and von Jagow (1987). These comprised a $10 \times 16 \times 0.15$ -cm "resolving gel" polymerized from a solution containing 12.5% (wt/vol) acrylamide with 3% cross-linker underlying a $2 \times 10 \times 0.15$ -cm "spacing" gel containing 10% (wt/vol) acrylamide and 3% cross-linker, and a $2 \times 10 \times 0.15$ -cm "concentrating" gel. Electrophoresis was carried out for 16 h at 75 V, and the gel then briefly stained and destained, dried under vacuum, and exposed to Fuji RX x-ray film at -70°C.

Immunoprecipitations

Labeled cells were lysed in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% (wt/vol) Triton X-100, 5 mM EGTA, 2 mM MgCl₂, 1 mM PMSF, 50 µM leupeptin, 50 µM E-64, 10 µM pepstatin A, and 10 µg/ml chymostatin (lysis buffer) on ice for 45 min (final concentration 10⁷ cells/ml), and then centrifuged at 13,000 g for 15 min. The resulting supernatants were removed, recrystallized BSA (final concentration 5 mg/ml) and either monoclonal QE11 or 312H (final dilution 1:100 [vol/vol]) added, and the solutions incubated at 4°C for 2-4 h with constant mixing. Rabbit anti-mouse Ig Sepharose was then added (250 µl of a 50% suspension in lysis buffer per milliliter of lysate) and the resulting suspension incubated for a further 12-16 h. The immunoabsorbent was then collected by centrifugation (2 min at 2,000 g), washed three to five times with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.5% Triton X-100, and bound proteins eluted by incubation for 30 min at 40°C in SDS sample buffer. The eluates were then fractionated by SDS-PAGE as described above.

Results

Binding and Endocytosis of Specific Antigen

Incubation of cell-line 11.3 with radiolabeled antigen at 0°C resulted in saturable binding, consistent with the expression

of $\sim 1 \times 10^4$ receptors per cell. Upon incubation at 37°C the bound antigen was endocytosed at an initial rate of 3.3% per min. The proportion of protease resistant (intracellular) radiolabel reached a maximum after 45–60 min and thereafter declined, whereas the amount remaining on the cell surface continued to decrease for at least 8 h (Fig. 1). After 15–20 min TCA soluble radiolabel was detected in the incubation medium, and this continued to increase for the remainder of the incubation period, such that after 8 h $\sim 50\%$ of the antigen initially bound had been rendered acid soluble. Such kinetics are similar, albeit slightly slower, to those observed with other tetanus toxin/toxoid specific B cells (Watts and Davidson, 1988).

In addition to acid-soluble radiolabel, TCA-precipitable antigen was also released into the incubation medium upon rewarming to 37°C, although in this case no lag was observed (*inset*, Fig. 1). To analyze this material by gel electrophoresis it was necessary to perform the incubations at 37°C in protein-free medium. Under these conditions the antigen was endocytosed and degraded in a manner indistinguishable from that observed in the control incubations. Examination of the TCA-precipitable radiolabel released into the medium by SDS-PAGE after chase incubations of up to 4 h revealed that $>95\%$ was intact antigen (data not shown), suggesting that most if not all, of this material is derived by dissociation from the receptor at the cell surface, or during recycling through peripheral endocytic compartments.

Time-dependent Fragmentation of Antigen

The actual size of presented antigen fragments is not yet known although peptides as small as 7–17 amino acids in length can elicit MHC-restricted T cell responses *in vitro* (reviewed by Buus et al., 1987). An electrophoresis system

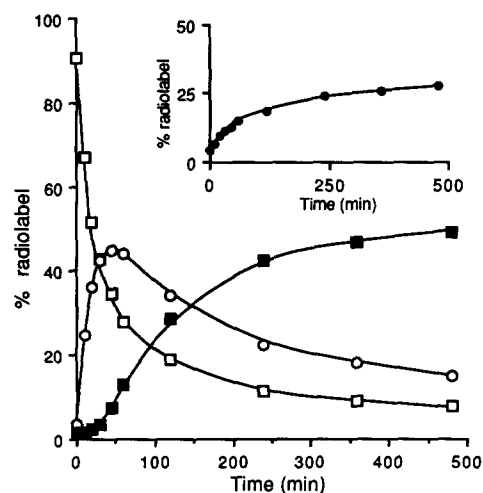


Figure 1. Endocytosis of prebound antigen. ^{125}I -Tetanus toxin was bound to clone 11.3 B cells at 0°C. After washing to remove unbound antigen, the cells were resuspended in B cell growth medium and chased at 37°C. Aliquots (2×10^6 cells) were removed at various times and maintained on ice until the end of the chase period. Intracellular (○) and cell surface (□) radiolabel was determined after digestion of the cell pellet with pronase, and acid soluble radiolabel (■) after TCA precipitation of the medium. TCA-precipitable radiolabel present in the medium (representing dissociated antigen) is shown in the inset. All results are expressed as a percentage of the antigen initially bound.

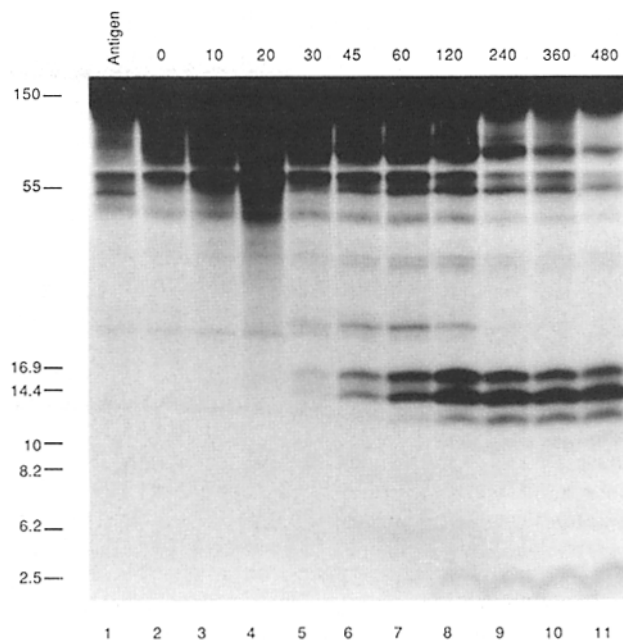


Figure 2. Time-dependent fragmentation of antigen. B cells (clone 11.3) were incubated with ^{125}I -antigen at 0°C. After washing to remove unbound antigen, the labeled cells were resuspended in B cell growth medium and chased at 37°C for times as indicated. Aliquots (2×10^6 cells) were then collected by centrifugation, resuspended in SDS sample buffer, and fractionated by electrophoresis as described in Materials and Methods. Antigen fragments were detected by autoradiography, and their apparent molecular weights determined by reference to standard proteins (Sigma SDS-7 and SDS-17).

designed for the optimum separation of proteins in the molecular mass range 2–50 kD (Schägger and von Jagow, 1987) was used to investigate the fate of endocytosed antigen. To achieve the highest resolution, and to allow analysis of proteins of higher molecular weight, an additional separating gel was included between the “concentrating” and “resolving” gels.

In the absence of reducing agents the radiolabeled antigen comprised primarily ($>95\%$) a single component of M_r 150 kD (Fig. 2, lane 1). Minor components of M_r 100, 50, 20, and 13 kD, which could not be resolved from the intact antigen by gel filtration chromatography, were also present. With the exception of the 50-kD fragment, these proteins were also specifically bound by clone 11.3 B cells at 0°C (Fig. 2, lane 2).

Cells to which radiolabeled antigen had been bound at 0°C, were incubated at 37°C for various times and the composition of the total cell-associated antigen analyzed by SDS-PAGE. This analysis revealed the time dependent production of discrete fragments (Fig. 2, lanes 2–11). Degradation of the antigen could not be detected for the first 10–20 min of the chase, but thereafter proceeded as a pseudo first order reaction with an apparent half-time of ~ 2 h. Initially, fragments of M_r 55, 30, 21, 16, and 14 kD were produced (lanes 4–6). However the fates of individual products varied, for example, the 21-kD fragment reached a maximum after 1 h and thereafter declined, while the 14-kD fragment continued to accumulate throughout the chase incubation. After 8 h the major radiolabeled degradation products associated with this

clone were proteins of *M*, 55, 16, 14, 12.5, and 2.5 kD and together accounted for ~10–15% of the radiolabeled antigen initially bound.

Inhibition of Antigen Fragmentation

Several previous studies have demonstrated that perturbation of the endosome/lysosome pathway can also inhibit MHC class II restricted antigen presentation (e.g., Ziegler and Unanue, 1982; Streicher et al., 1984). We therefore investigated the effects of selected inhibitors on antigen degradation by specific B cells both in terms of the liberation of acid soluble radiolabel (Table I), and the generation of specific fragments (Fig. 3).

In each case the results obtained were consistent with "late" endosomes and lysosomes being the site of antigen degradation. Thus incubation of cells in the presence of metabolic inhibitors or at 20°C (which prevents delivery to, or maturation of, "late" endosomes/lysosomes [Dunn et al., 1980]) both completely abolished fragmentation (Table I, Fig. 3, lanes 2 and 9) although endocytosis of antigen still proceeded, albeit more slowly, at 20°C (Table I and data not shown). Similarly, incubation in media containing the weak base chloroquine or inhibitors of lysosomal thiol proteases resulted in a dose-dependent inhibition of the release of TCA soluble radiolabel (Table I), and distinct fragmentation patterns (Fig. 3, lanes 4, 5, and 7). In contrast, the cathepsin D inhibitor pepstatin A did not influence the fragments observed (Fig. 3, lane 6) despite inhibiting the release of acid soluble radiolabel. In every case, agents which directly inhibited proteolysis caused an accumulation of intracellular antigen compared to control cells (Table I).

Table I. Effect of Selected Inhibitors on Antigen Processing

Inhibitor		Percent acid soluble	Percent intracellular
	μM		
Leupeptin	100	58.7 \pm 1.2	166 \pm 17
	200	53.0 \pm 3.5	179 \pm 16
	500	44.0 \pm 3.2	195 \pm 19
E-64	100	68.0 \pm 4.1	145 \pm 10
	200	57.8 \pm 1.6	172 \pm 9
	500	42.6 \pm 2.0	203 \pm 18
Pepstatin A	100	93.6 \pm 2.4	99 \pm 1
	200	90.1 \pm 0.8	106 \pm 3
	500	84.4 \pm 2.9	108 \pm 5
Chloroquine	100	46.5 \pm 1.0	157 \pm 28
	200	38.8 \pm 2.4	155 \pm 25
	500	29.1 \pm 0.7	129 \pm 11
Metabolic inhibition		Not detectable	Not detectable
20°C Chase		Not detectable	91 \pm 8

In general B cells (clone 11.3) were incubated for 1 h at 37°C in growth medium containing inhibitors as indicated. Cells were collected by centrifugation, and ^{125}I -antigen bound at 0°C. After extensive washing to remove unbound antigen, the labeled cells were resuspended in medium containing inhibitors as appropriate and chased for 4 h at 37°C. For experiments involving metabolic inhibition, PBS containing 1 mM MgCl_2 , 0.5 mM CaCl_2 , 5 mg/ml BSA, and either 20 mM glucose or 20 mM Na azide + 50 mM 2-deoxyglucose was used instead of growth medium, and chase incubations were for 90 min. In experiments using chloroquine, antigen was bound to cells not previously preincubated with inhibitor, while in chase incubations performed at 20°C the medium also contained 20 mM Na-Hepes, pH 7.4. All results are expressed relative to control incubations and are the mean \pm SEM of at least three separate determinations.

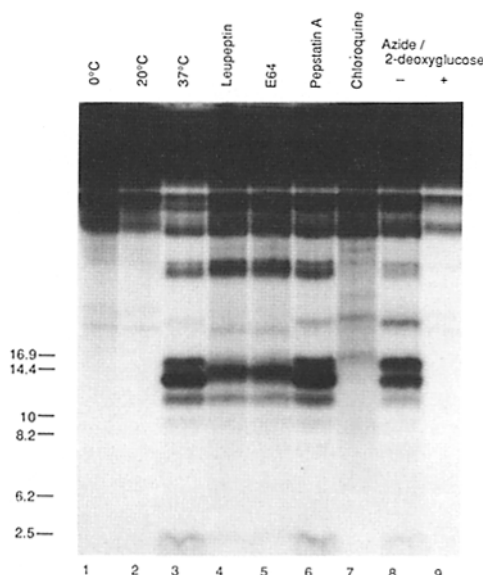


Figure 3. Inhibition of antigen fragmentation. B cells (clone 11.3) were treated with inhibitors as described in the legend to Table I. Cell pellets (2×10^6 cells) from incubations with the highest concentrations of inhibitors used were homogenized in SDS sample buffer and antigen fragments detected by electrophoresis and autoradiography.

Immunoprecipitation of mIgG–Antigen Complexes

Since we have shown that mIgG–antigen complexes remain tightly associated at mildly acidic pH (Watts and Davidson, 1988) we predicted that fragmentation of the antigen might begin while it remained bound to the mIgG. To test this hypothesis we immunoprecipitated mIg from cells which had processed radiolabeled antigen for various periods of time. Using a monoclonal antibody recognizing the kappa light chain (expressed by clone 11.3) almost quantitative precipitation of antigen fragments was obtained at all chase times (Fig. 4, lanes 1 and 3–5), whereas virtually no radiolabel was precipitated from parallel incubations using an anti-lambda monoclonal (lanes 2 and 6). These results demonstrate that fragmentation does indeed commence while the antigen is associated with mIgG, and suggest that the B cell epitope is formed by some or all of the fragments observed.

Epitope-specific Fragmentation

Since the results described above demonstrate that the initial substrate for antigen processing in specific B cells is the antigen–mIg complex we tested the hypothesis that the epitope through which the antigen is bound might directly influence the fragmentation profile observed. Radiolabeled antigen was bound at 0°C to several B cell clones which recognize different epitopes within the tetanus toxin molecule (Lanzavecchia, 1986; Demotz et al., 1989). After incubations at 37°C for 2 h the total cell-associated radiolabeled antigen was analyzed by SDS-PAGE and autoradiography. For each cell line shown (and three others also tested) a reproducible pattern was observed consisting of a unique combination of common and "cell-restricted" fragments (Fig. 5). For example, each cell generated fragments of *M*, 35 and 33 kD, although the relative efficiencies of formation and/or degrada-

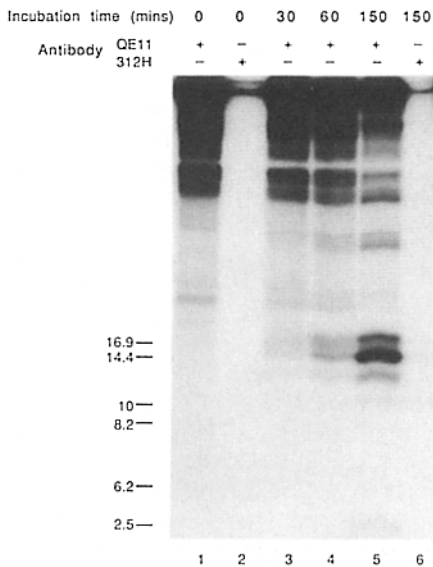


Figure 4. Immunoprecipitation of receptor-bound antigen. B cells (clone 11.3) were incubated with ^{125}I -antigen at 0°C . After washing to remove unbound antigen, the labeled cells were resuspended in B cell growth medium and incubated at 37°C for times as indicated. Aliquots (2×10^6 cells) were collected by centrifugation and lysed in lysis buffer. Specific (QE11) or nonspecific (312H) antibody was then added, and human IgG immunoprecipitated. Receptor-bound antigen was eluted with SDS sample buffer, fractionated electrophoretically, and visualized by autoradiography.

tion varied. Similarly, clones A46 and 8.5 both produced fragments of M_r 20, 15, and 4 kD albeit to differing extents, whereas clone 11.3 did not accumulate significant amounts of any of these products. Both 8.5 (e.g., M_r 31, 28, 11, and 8 kD) and 11.3 (e.g., M_r 16, 14, 12.5, and 10 kD) produced several "cell-restricted" fragments whereas the profile generated by clone A46 was much simpler (Fig. 5). It must be

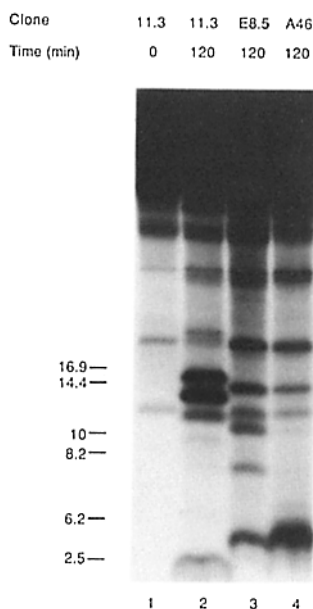


Figure 5. Comparison of antigen processing by clonal B cell lines. B cells (clones 11.3, 8.5, and A46) were incubated with ^{125}I -antigen at 0°C . After washing to remove unbound antigen, the cells were resuspended in B cell growth medium and incubated at 37°C for 2 h. Cell pellets were homogenized in SDS sample buffer and antigen fragments visualized after electrophoresis and autoradiography.

remembered that by using this method of analysis we are only able to visualize iodinated fragments which are likely to be a subset of those actually generated. Nonetheless the results known in Fig. 5 clearly demonstrate differential antigen processing by clonal B cells.

Influence of Heterologous Fab Fragments on Antigen Processing

Fab fragments derived from heterologous monoclonal anti-tetanus toxin antibodies can bind specifically to other accessible epitopes on cell-associated antigen (Watts and Davidson, 1988). Since the results presented above strongly suggested that the epitope through which the antigen is bound directly influences proteolysis, we thought it possible that Fab-antigen-mIg complexes would be processed in a distinct fashion after endocytosis. The results of an experiment to test this hypothesis are shown in Fig. 6. For three different B cell clones tested incubation with the homologous Fab had no effect on the fragmentation profile (Fig. 6, lanes 2 and 3, 6 and 8, and 10 and 13) consistent with their inability to bind to monovalent antigen specifically bound to the cell surface (Watts and Davidson, 1988). However, in most instances heterologous Fabs had a striking effect on the fragmentation patterns. For example, processing of 11.3 Fab-antigen complexes by either clone A46 or clone 8.5 resulted in the production of "11.3 specific" fragments of M_r 14, 12.5, 10, and 2.5 kD (Fig. 6, lanes 4 and 12). However the new fragmentation profiles were not simply the sum of the patterns charac-

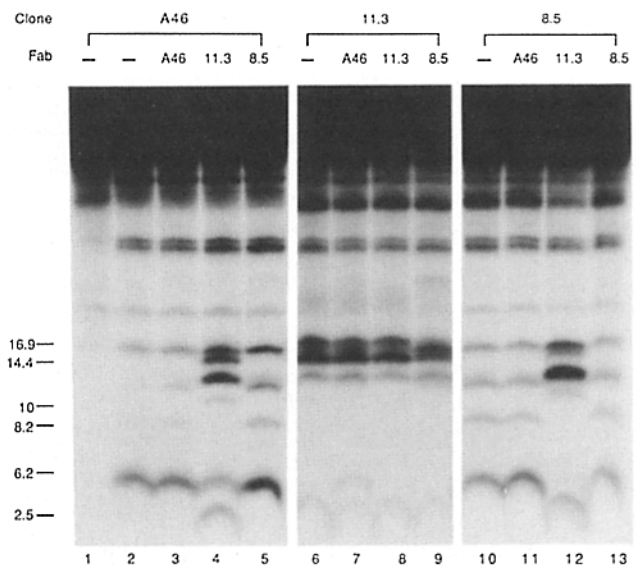


Figure 6. Effect of Fab fragments on antigen processing. B cells (clones A46, 11.3, and 8.5) were incubated with ^{125}I -antigen at 0°C . After washing to remove unbound antigen, the labeled cells were resuspended at a density of 2×10^7 cells/ml in either B cell growth medium alone, or medium containing $25 \mu\text{g/ml}$ unlabeled Fab fragments prepared from anti-toxin antibodies secreted by clones A46, 11.3, or 8.5 and incubated at 0°C for 90 min. The suspensions were then diluted with medium to a final concentration of 4×10^6 cells/ml, and incubated at either 0°C (lane 1) or 37°C (lanes 2-13) for 2 h. Cell associated antigen fragments (2×10^6 cells) were detected after electrophoresis and autoradiography.

teristic of the two antibody specificities involved; the presence of the 11.3 Fab prevented the accumulation of an 8-kD fragment normally generated by both A46 and 8.5 cells (Fig. 6, lanes 4 and 12). Moreover, the ability of the B cell clones to accumulate material with M_r 4 kD was differentially affected; clone A46 continued to accumulate material of this size in the presence of 11.3 Fabs while clone 8.5 did not. Similarly, processing of A46 Fab-antigen complexes but not 8.5 Fab-antigen complexes by clone 11.3 resulted in production of a 4-kD fragment not normally generated by this clone. Instead, in 11.3 cells, the principal effect of the 8.5 Fab appeared to be to suppress accumulation of the 16-kD fragment normally produced and enhance the accumulation of a 15-kD fragment (Fig. 6, lanes 7 and 9). Taken together, the results of this experiment confirm that it is the epitope specificity of the mIgG, rather than other clonal differences, which is primarily responsible for the fragmentation patterns characteristic of each clone.

Discussion

Although several *in vitro* studies have demonstrated that protein denaturation may be sufficient to permit functional MHC class II-antigen-T cell receptor complexes to be formed (e.g., Streicher et al., 1984), and there are instances in which native proteins can be presented by aldehyde-fixed APCs (Allen, 1987), probably the most relevant form of antigen processing *in vivo* is intracellular proteolysis. However, at present the precise molecular details of this process are still poorly understood, although it is generally accepted that it occurs within the endosome/lysosome pathway (see the introduction of this article).

Many previous studies of cellular antigen processing have investigated the class II restricted presentation of small globular proteins such as myoglobin, cytochrome *c*, or lysozyme, using macrophages as APCs (reviewed by Unanue, 1984; Allen, 1987). In such cases the APCs do not possess specific antigen receptors. Consequently internalization is primarily restricted to "fluid phase" endocytosis, and accumulation limited by the concentration of the antigen in the incubation medium. In contrast, uptake by antigen-specific B lymphocytes or lymphoblastoid cell lines is a receptor-mediated process, which permits efficient presentation at antigen concentrations three to four orders of magnitude lower than those required by nonspecific cells (Rock et al., 1984; Lanzavecchia, 1985; Casten and Pierce, 1988). In this case, the internalization of a relatively stable receptor-ligand complex almost certainly dictates that antigen processing, at least at its earliest stages, is a uniform process within individual clonal cell lines; the orientation of the antigen, and hence the regions most accessible to, or protected from, the actions of proteolytic enzymes will be constant. This system therefore offers several experimental advantages in the study of cellular antigen processing.

Several lines of evidence support the hypothesis that the B cell-derived antigen fragments described here are produced at the same time and in the same compartments as those which are actually presented in MHC-restricted form. For example, previous studies have demonstrated that there is a lag of ~ 1 h between the initial exposure of APCs to antigen, and the time at which they are first able to stimulate specific T cells (e.g., Unanue, 1984; Roosnek et al., 1988). This presumably reflects the time taken for delivery of the

antigen to the processing compartment, processing itself, and retrieval and transport of the relevant peptides to the cell surface. Thus the fact that several of the fragments presently described can be detected within 30 min of the start of the chase period (Fig. 2) is consistent with their being generated in the immunologically relevant compartment. Similarly, the effects of the various inhibitory conditions tested, for example chase incubations at 20°C or in the presence of chloroquine (which inhibit both fragmentation and presentation [Lanzavecchia, 1985; our unpublished results]), support the hypothesis that the two processes are directly related.

Despite this apparently strong correlation, we cannot yet be certain of the precise immunological relevance of any of the fragments observed. The fact that the patterns are strikingly different suggests that each clonal B cell line processes the antigen in a unique fashion. The variations observed cannot be attributed simply to genetic differences (since two of the cells shown are from the same donor), nor to the differential expression of proteolytic enzymes by individual clones (since heterologous "cell-restricted" fragments can be induced by treatment with appropriate Fabs; Fig. 6), and must therefore reflect the direct influence of the mIg. The demonstration that almost all of the fragments can be quantitatively immunoprecipitated using a specific monoclonal anti-human IgG antibody might suggest that they are prevented from associating with the MHC glycoproteins by virtue of their association with the mIg. However given the relatively large size of many of the fragments when compared to minimal peptides capable of eliciting T cell responses *in vitro*, we believe that some of them might also bind to MHC molecules, particularly since a previous study demonstrated that presentation of myoglobin-anti-myoglobin-anti-mIg complexes by nonspecific B cells was not adversely affected if the T cell and anti-myoglobin epitopes did not coincide (Ozaki and Berzofsky, 1987). Thus binding of the fragments to the MHC glycoproteins might occur either after further processing, or after dissociation from the mIg, or even as tripartite complexes in association with the mIg. Indeed it is possible that the persistence of the fragments within the cell might greatly favor their association with class II MHC molecules over other parts of the antigen which are more rapidly degraded, particularly if the association rates *in vivo* are as slow as those calculated from *in vitro* studies (Buis et al., 1986). However, to date we have been unable to demonstrate any association between the major proteolytic fragments observed and the class II glycoproteins expressed by these cells.

There are several instances where there appear to be preferential pairings among T and B cell clones specific for epitopes within the same antigen. For example, it has been observed that in the tetanus system particular T cell clones are preferentially stimulated when antigen capture and endocytosis is mediated by certain B cell clones (Lanzavecchia, 1986). Additionally, B cells, by virtue of the antibodies they secrete, can directly influence the expressed T cell repertoire as illustrated by the differential stimulation of individual T cell clones by nonspecific APCs exposed to defined monoclonal antibody-antigen complexes (Ozaki and Berzofsky, 1987; Manca et al., 1988). Conversely, "help" for the production of particular antibody specificities is more readily provided by some T cell clones than others (Manca et al., 1985). In other words antigen specific T and B cells may influence each other's expressed repertoire in a reciprocal fashion (Berzofsky, 1983). Berzofsky has also suggested that

such T-B reciprocity might be the result of immunoglobulin-directed proteolytic processing within the B cell; our results provide the first direct evidence for this in clonal B cell lines. We propose that such differential processing results in the generation of distinct spectra of MHC-restricted peptides, and is therefore directly responsible for differential T cell recruitment and activation by B cells with different epitope specificity. In addition, as processing within a single B cell clone can be perturbed when additional epitopes are complexed this suggests that antigen processing within B lymphocytes will also be affected by preexisting antibodies reactive with other epitopes.

Although unique antigen-mIg complexes are internalized by individual B cell clones, each having distinct conformations and ligand affinities, they presumably all follow a common intracellular itinerary. It appears that, unlike many other receptor-ligand complexes, endocytosed antigen-mIgG can follow more than one major route. Previously we demonstrated that, in common with other receptor-ligand complexes stable at acidic pH such as the transferrin receptor (Dautry-Varsat et al., 1983; Klausner et al., 1983), a substantial proportion of endocytosed antigen-mIgG is recycled to the cell surface (Watts and Davidson, 1988). However, in contrast to transferrin, which dissociates at the cell surface, and to ligands such as LDL and asialoglycoproteins, which dissociate from their receptors within endosomes (reviewed by Wileman et al., 1985), the results presently described clearly demonstrate that a significant proportion of the antigen is degraded in association with its receptor.

The question remains whether recycling and degradation are mutually exclusive pathways (i.e., do antigen-mIgG complexes recycle through protease containing compartment[s]?). To date we have been unable to detect any cell-derived antigen fragments associated with the plasma membrane (data not shown). We therefore favor the hypothesis that passage of antigen-mIgG complexes into the protease containing compartment is essentially unidirectional, and that recycling involves a more peripheral compartment. This conclusion appears consistent with the results of a study which suggested that transferrin is generally confined to "early" endosomes (Schmid et al., 1988), and others which indicated that transport of dissociated ligands to their site of degradation involves a prelysosomal compartment from which the relevant recycling receptors are excluded (Mueller and Hubbard, 1986; Stoorvogel et al., 1987).

The suggestion that mIgG does not recycle from protease containing compartments would also predict that the receptor itself is degraded, in a situation analogous to occupied growth factor receptors such as the EGF receptor (Beguinot et al., 1984). This hypothesis is consistent with the observation that in the absence of protein synthesis, the B cell's capacity to accumulate antigen is limited (Watts and Davidson, 1988). However, it must be remembered that the immunologically relevant products of antigen processing are retrieved and transported to the cell surface. This probably involves receptors other than mIg (such as the MHC glycoproteins) but it is apparent that some mechanism for recycling from protease containing compartments must exist.

We are particularly grateful to Dr. Antonio Lanzavecchia, Basel Institute for Immunology, for providing the cell lines used in this work and for valuable discussions. We also thank Dr. N. Fairweather and Mr. A. Sheppard, Wellcome Biotech, for gifts of tetanus toxin and toxoid; Drs. R. Jefferis,

S. van Heyningen, and P. Jackson for advice; and our colleagues for discussions and criticism of the manuscript.

This work was supported by a Medical Research Council Project grant.

Received for publication 1 February 1989 and in revised form 17 March 1989.

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