The EMBO Journal vol.7 no.7 pp.1937 - 1945, 1988

Endocytosis and recycling of specific antigen by human B cell lines

Colin Watts and Howard W.Davidson

Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, UK

Communicated by M.J.Crumpton

Human B cell lines expressing membrane immunoglobulin specific for tetanus toxoid/toxin were used to study the receptor-mediated endocytosis of antigen. Monovalent antigen, initially bound to cell surface immunoglobulin at 0°C, was rapidly endocytosed upon warming the cells to 37°C. The kinetics of endocytosis of antigen were independent of the number of occupied binding sites and indicated a half-life for antigen on the cell surface of 8.5 min. Endocytosis of antigen apparently ceased after ~15 min at 37°C, although some 40–50% remained on the cell surface at this time. We show, using biotinylated antigen and an avidin detection assay, that this is due to recycling of antigen to the cell surface. By labelling the antigen on the cell surface with Fabs against different epitopes we show that antigen continues to be endocytosed for at least 1 h after the initial rapid phase of endocytosis, again indicating that there must be recycling of immunoglobulin/antigen complexes. As a consequence of the stable interaction between antigen and membrane immunoglobulin, the capacity of the cells to accumulate antigen was limited when the synthesis of membrane immunoglobulin was blocked; under these conditions only 2–3 times as much antigen was endocytosed and degraded when antigen was supplied continuously over a 4-h period at 37°C as could be bound to the cells at 0°C. These results reveal a rapid and efficient pathway for the endocytosis and recycling of monovalent antigen in B cells.

Key words: endocytosis / antigen processing / membrane immunoglobulin/membrane recycling

Introduction

To mount an effective immune response both B and T cells specific for a particular antigen are required. However, whereas B cells recognize features of native antigen via their membrane immunoglobulin (mIg), T cells preferentially recognize a complex formed between antigen and the MHC glycoproteins. This restriction dictates that antigen must be recognized on the surface of another cell and that it must be processed by proteolysis and/or denaturation in order to generate the correct structural features for binding to the MHC glycoproteins and to the T cell antigen receptor (reviewed in Unanue, 1984; Grey and Chesnut, 1985; Germain, 1986; Allen, 1987; Bevan, 1987; Parham, 1988).

The realization that antigens must generally be metabolized before MHC-restricted recognition by T helper cells can occur came initially from studies using macrophages as antigen-presenting cells. The crucial observations were that, after the initial binding of antigen, there was a lag of ~1 h before it could be recognized by T cells and that the events occurring within the presenting cell during this time were sensitive to weak bases and to fixation with glutaraldehyde (Ziegler and Unanue, 1982).

Another important early finding was that although aldehyde fixation blocked presentation of native antigen, tryptic fragments of antigen could, in some cases, be presented in an MHC-restricted fashion by fixed cells (Shimonkevitz *et al.*, 1983). Thus, different fragments of an antigen could be tested for their ability to interact with particular MHC Class II haplotypes and, as a complex, to activate antigen-specific T cell clones. This approach, which bypasses the processing events that the native antigen must normally undergo, has become increasingly sophisticated and has now reached a point where short peptides derived from well defined antigens are being used, together with variants carrying single amino acid substitutions, in order to define the requirements for effective binding to the MHC Class II glycoproteins and, as a complex, to activate antigen-specific T cell clones (Babbitt *et al.*, 1985; Buus *et al.*, 1986; Allen *et al.*, 1987; Guillet *et al.*, 1987; Sette *et al.*, 1987; Rothbard *et al.*, 1988).

Such observations suggest a crucial role for events occurring within the cell during the normal handling of antigen by presenting cells, and raise several important questions. For instance, what happens to native antigen when the requirement for metabolism within the presenting cell is not bypassed? What are the steps between the initial binding of native antigen to the presenting cell surface and its subsequent reappearance in a processed form associated with MHC glycoproteins? What role do the pathways of endocytosis and membrane recycling play in antigen processing and presentation?

T cells, initially activated by recognition of processed antigen on the surface of a macrophage or dendritic cell, can now provide 'help' to B cells displaying the same processed, MHC-restricted form of the antigen on their cell surface. However, in the case of the B cell the initial recognition of native antigen is via specific mIg, and because recognition of native antigen by specific B cells is 'receptor mediated', the levels of antigen required for subsequent T cell recognition of the processed form are several orders of magnitude lower than are required for non-specific presenting cells (Chesnut and Grey, 1981; Rock *et al.*, 1984; Lanzavecchia, 1985). To analyse the biochemical details and the cell biological features of antigen endocytosis and processing, the 'receptor-mediated' system offered by specific B cells is the one of choice.

Several Epstein–Barr virus (EBV)-transformed human B cell lines specific for tetanus toxoid have recently been isolated that present antigen to specific T cells in an MHC-restricted fashion (Lanzavecchia, 1985, 1987). The characteristics of presentation mirror those observed with other

types of presenting cell, i.e. presentation is demonstrable 45–60 min after the initial introduction of antigen but is blocked by treatment with chloroquine or by fixation of the cells during, but not after this lag period (Lanzavecchia, 1985). We have begun to analyse the endocytosis and processing of specific antigen using these cells. We show here that monovalent antigen initially bound to mIgG is rapidly endocytosed but that the majority of antigen/mIg complexes are subsequently returned to the cell surface and then cycle to and from intracellular compartments. None the less, the ultimate fate of the antigen is extensive degradation.

Results

Specific binding of antigen to human B cell lines

The capacity of EBV-transformed cell lines to bind antigen at 0°C was assessed by incubating cells expressing mIgG specific for tetanus toxoid with various levels of purified, radiolabelled tetanus toxin in the presence or absence of an excess of unlabelled tetanus toxoid. Another EBV-transformed line (ALCL-3) which also expresses mIgG but of an unknown specificity was tested as a control. Rather than use the toxoid (formaldehyde-treated toxin) as antigen, we decided to use the native antigen in order to facilitate the isolation of monovalent material. As shown in Figure 1 only the specific lines E8.5 and A46 (and three others tested) bind the antigen in a saturable fashion demonstrating specific binding to the mIgG expressed by these cells. At 0°C these receptors are saturated at ~2 μg/ml antigen. Specific binding to the cell surface was not due to secretion of IgG and subsequent rebinding via Fc receptors since ALCL-3 cells did not acquire specific binding sites for tetanus toxin after incubation at 37°C for 1 h in medium used to grow clone E8.5 (which contains ~5 μg/ml secreted IgG). Nor did clone E8.5 cells lose any specific binding capacity after a similar incubation in ALCL-3 growth medium.

The atoxic C fragment, a 47-kd portion of the heavy chain of tetanus toxin generated by papain cleavage (Helting and Zwisler, 1977), also bound specifically to the mIg on E8.5 cells but not to A46 cells, demonstrating that the latter cell line recognizes an epitope elsewhere in the 150-kd toxin molecule. This turned out to be a useful feature, allowing us to examine both the kinetics of endocytosis of different forms of the antigen and to address the question of recycling of antigen (see below). Unless it is specifically stated that the C fragment has been used, 'antigen' refers to intact tetanus toxin. Binding was inhibited by >90% in the presence of excess unlabelled tetanus toxoid.

Endocytosis of pre-bound antigen

Cells to which radiolabelled antigen had been specifically bound at 0°C were warmed to 37°C for various times, rechilled to 0°C and then collected by centrifugation. The supernatant, containing any antigen returned to the medium, was retained. Endocytosed antigen was distinguished from antigen remaining on the cell surface by digestion of the resuspended cells with 1.5 mg/ml pronase at 0°C for 1 h or by washing the cells at pH 1.7. The treated cells were collected by centrifugation and radioactivity was assayed in the supernatant and pellet fractions.

Warming the cells to 37°C resulted in a rapid clearance of antigen from the cell surface (Figure 2). The initial rate of endocytosis of antigen was 8% or 5%/min of that initially bound to cell lines E8.5 and A46 respectively. Assuming first-order kinetics, these rates indicate a half-life of antigen on the cell surface of ~8.5 or ~13 min (E8.5 and A46 respectively). We consistently observed a faster initial rate of endocytosis on line E8.5 whether native toxin or the C fragment (Figure 2) was used as antigen. After 15 min the amount of antigen sequestered within the cells reached a maximum and thereafter declined. At this time only 4% of the antigen had dissociated or had been otherwise returned to the medium.

Since many earlier studies had demonstrated that cross-linking leads to rapid patching, capping and clearance of mIg from the B cell surface (Taylor *et al.*, 1971; Loor *et al.*, 1972), we wanted to rule out the possibility that our monovalent antigen preparation had become aggregated as a result of radiolabelling and was inducing cross-linking and endocytosis of mIg. We therefore rechromatographed the radiolabelled antigen by gel filtration chromatography. Greater than 99% of the radiolabel was eluted with an apparent mol. wt of 150 000, thus eliminating the possibility that multivalent antigen complexes were responsible for the rapid rate of endocytosis.

Another point of concern was the grossly non-physiological situation created by saturating all the mIg with antigen. *In vivo* a B cell would probably never encounter

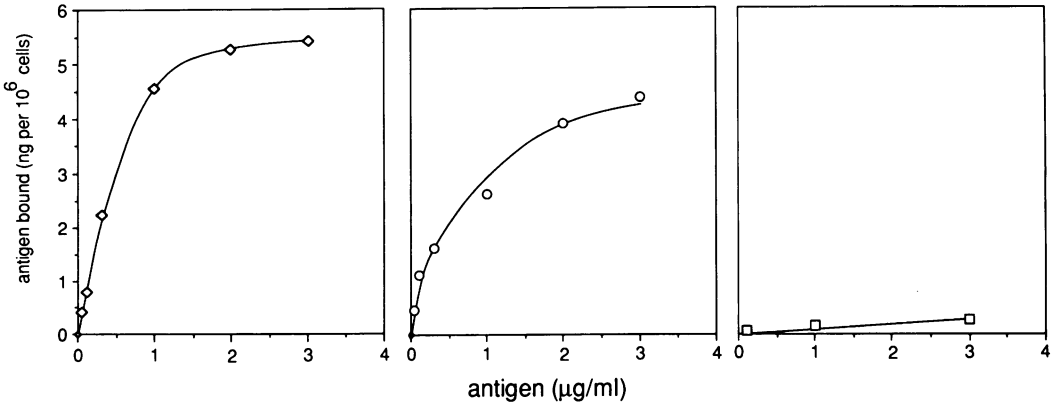

Fig. 1. Binding of ^{125}I-labelled antigen to EBV-transformed B cell lines. Cell lines A46 (◇), E8.5 (○) or ALCL-3 (□) were incubated at 0°C as described in Materials and methods with different concentrations of radiolabelled antigen in the presence or absence of excess unlabelled antigen. The cells were washed twice by centrifugation and resuspension and radiolabel remaining bound was assayed by gamma counting. Binding was reduced 10- to 20-fold in the presence of excess unlabelled antigen and this non-specific binding has been subtracted from the plotted data.

levels of specific antigen as high as 2 μg/ml. Since under physiological conditions only a small proportion of the available mIg would be occupied, we examined the kinetics of endocytosis of antigen at various levels of receptor occupancy ranging from saturation down to a point when < 1 in 10 available binding sites were occupied. The results in Figure 3 show clearly that antigen is endocytosed with the same kinetics whatever the number of occupied receptors. Although it is difficult, for technical reasons, to measure the initial rate of endocytosis of antigen much below a level of 10% occupied receptors, the low concentrations of antigen used to achieve this level of occupancy (10^{-10} M for 7% occupancy) are physiologically relevant, being sufficient to give maximum stimulation of T cells by tetanus toxoid specific B cells but no stimulation by non-specific B cells (Lanzavecchia, 1985, 1987). This capacity for efficient and rapid endocytosis of antigen must be a key factor enabling specific B cells to become engaged in collaborations with specific T cells at very low levels of antigen.

The extended time courses shown in Figure 2 demonstrate that after 20–30 min of incubation at 37°C the cells began to return radioactivity to the medium. This increased progressively over the next 2 h whilst the level of antigen associated with the cells began to decline. After 2 h between 30 and 50% (depending on the cell line) of the antigen, initially bound to mIg on the cell surface, had been returned to the medium. Of this material only 30% was precipitated by trichloroacetic acid (TCA) whereas 95% of the radiolabelled antigen was TCA precipitable at the beginning of the experiment. Our interpretation of these results is that extensive proteolytic degradation occurs within the cells, and that much of this material is returned to the medium. Greater than 95% of the TCA-precipitable material in the medium, when analysed by SDS–PAGE, was found to be intact antigen, whereas gel analysis of the cell-associated radioactivity revealed that discrete fragments of antigen were generated in a time-dependent manner during the incubation at 37°C (data not shown). The immunological relevance of these fragments remains to be determined.

Consistent with the idea that antigen was being degraded within the B cell endosome/lysosome system we found that when cells, with antigen pre-bound at 0°C, were chased at 37°C in the presence of 0.2 mM chloroquine, the amount of TCA-soluble radioactivity appearing in the medium was reduced to 25% of the control value. Similarly when the chase was performed at 20°C, endocytosis proceeded at 25% of the rate seen at 37°C but the reappearance of radioactivity in the medium was undetectable over a 4-h period (data not shown), which suggests that in B cells proteolytic degradation of endocytosed ligands is blocked at 20°C, a result previously observed in hepatocytes (Dunn *et al.*, 1980).

Is the endocytosis of antigen a strictly one-way process or are occupied antigen receptors recycled to the cell surface? We were prompted to ask this question because the amount of antigen that could be shown to be inside the cells at any one time never exceeded 40–60% of that initially bound. This could be explained if only a proportion of the cell surface receptors is capable of being rapidly endocytosed, the other occupied receptors remaining on the cell surface or being much more slowly endocytosed. Alternatively, endocytosed antigen might recycle to the cell surface so that

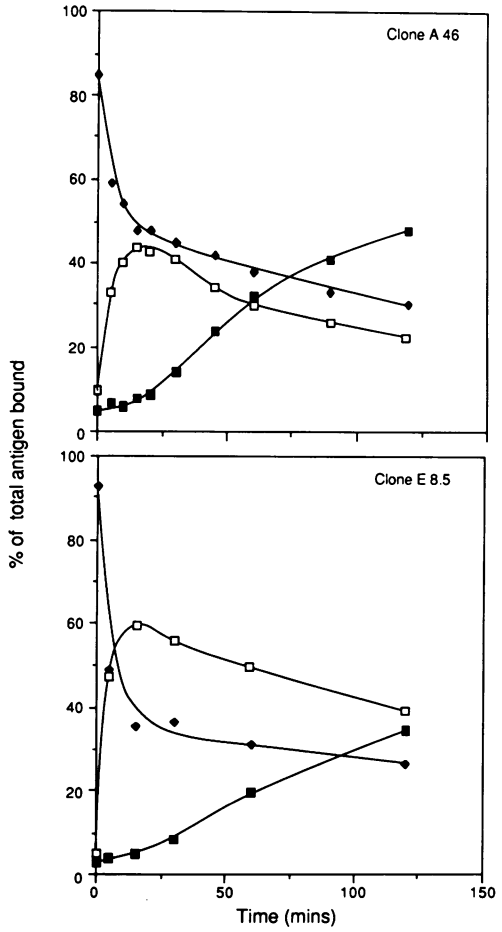

Fig. 2. Endocytosis of pre-bound antigen. The cell lines indicated were incubated at 0°C with labelled antigen (2.0 μg/ml tetanus toxin, **top panel**; 0.6 μg/ml C fragment, **bottom panel**), washed to remove unbound antigen and resuspended at 4 × 10^6 cells/ml as described in Materials and methods. Aliquots were warmed to 37°C for the indicated times, rechilled to 0°C and the cells and supernatant recovered by centrifugation. Radiolabelled antigen remaining on the cell surface (◆), sequestered within the cells (□) and that released into the medium (■) was determined as described in Materials and methods.

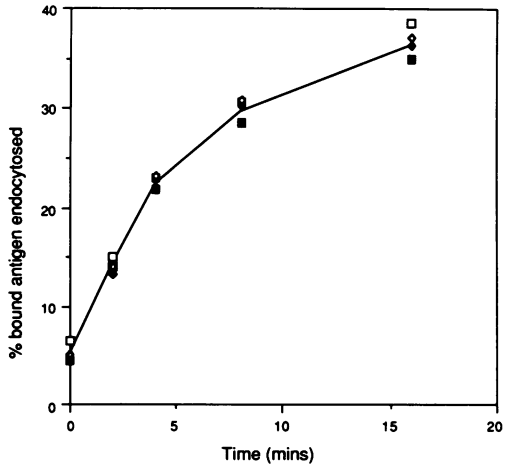

Fig. 3. Endocytosis of sub-saturating levels of bound antigen. Cells (A46) were incubated at 0°C with 1, 3, 9 or 26 × 10^{-10} M antigen. After washing free of unbound antigen this resulted in 7 (◇), 21 (■), 57 (◆) and 100% (□) respectively of the available binding sites becoming occupied at 0°C. Aliquots of each cell population were incubated at 37°C for the indicated times, rechilled and incubated with pronase as described in Materials and methods to assay endocytosed antigen.

after 20 min of incubation the rate of return of occupied receptors to the cell surface matches their rate of endocytosis, analogous to the behaviour of the transferrin receptor (Bleil and Bretscher, 1982; Hopkins and Trowbridge, 1983; Ciechanover *et al.*, 1983). To see if the kinetics observed in Figure 2 might be explained by antigen cycling to and from the cell surface we designed two experiments: one to determine whether endocytosed antigen is recycled to the cell surface, and another to measure the actual rate of antigen endocytosis at later time points, i.e. after the initial rapid redistribution.

Direct demonstration of antigen recycling

In order to show directly that a receptor recycles to the cell surface it is necesary to label receptors on the cell surface, allow them to be endocytosed and then follow their reappearance having first removed any receptors still present on the cell surface after the period allowed for endocytosis. In practice this is often achieved by proteolysis at 0°C. However, such a treatment may itself induce rapid exocytosis of internalized ligand/receptor complexes and undoubtedly does so in the case of the transferrin receptor on several human cell lines (our unpublished results). Since antigen bound to mIg could only be efficiently removed at pH values <2.0, which kills the cells, this method of assaying the reappearance of endocytosed antigen was also ruled out.

In order to circumvent these problems and to perform the experiment in a way which minimally perturbs the cells, we devised an assay to distinguish intracellular and cell surface antigen based on the preparation of biotinylated tetanus toxin and subsequent detection using [^{125}I]avidin. The characteristics of biotinylated antigen binding and endocytosis, assayed by measuring binding sites for [^{125}I]avidin, are shown in Figure 4. Saturation of cell surface receptors was achieved at ~0.5 μg/ml biotinylated antigen and binding was dramatically reduced in the presence of an excess of non-biotinylated tetanus toxoid. The kinetics of endocytosis of pre-bound biotinylated antigen, as detected by a loss of binding sites for [^{125}I]avidin, were similar to those observed for [^{125}I]-antigen (Figure 2) demonstrating that the assay is able to detect changes in the level of cell surface biotinylated antigen (Figure 4). Approximately 50% of the biotinylated antigen was endocytosed in a 20-min incubation at 37°C assuming that 4% dissociates into the medium during this time. When cells to which biotinylated antigen had been bound were pre-incubated with 10 μg/ml unlabelled avidin, subsequent binding of [^{125}I]avidin was reduced 10-fold to a background level observed on control cells never incubated with biotinylated antigen. This demonstrates that cell surface antigen can be effectively quenched, thus allowing the fate of endocytosed antigen to be followed independently of antigen remaining on the cell surface.

Cells (clone E8.5) were incubated with biotinylated antigen at 0°C, washed free of unbound antigen and split into two portions which were either held at 0°C or incubated at 37°C for 20 min to allow endocytosis to occur. Both sets of cells were then incubated first with unlabelled avidin at 0°C to quench any biotinylated antigen remaining on the cell surface and subsequently with 1 μM biotin to block unoccupied sites on avidin. Aliquots of the cell suspensions were reincubated at 37°C to allow membrane traffic to resume. After different lengths of time the cells were chilled and incubated at 0°C with [^{125}I]avidin to detect any biotinylated antigen on the cell surface. The results of the experiment are shown in Figure 5. Cells which had been allowed to endocytose the biotinylated antigen showed, upon rewarming after the incubation with unlabelled avidin, a time-dependent recovery in their ability to bind [^{125}I]avidin. In contrast there was no recovery of binding sites for [^{125}I]avidin on cells where the initial endocytosis of the antigen had been prevented. This control rules out the possibility that dissociation of the unlabelled avidin might account for the observed recovery or that the biotinylated antigen detected by the [^{125}I]avidin had not in fact been endocytosed but was for some other reason shielded from the unlabelled avidin and became, on rewarming, available for binding to the [^{125}I]avidin.

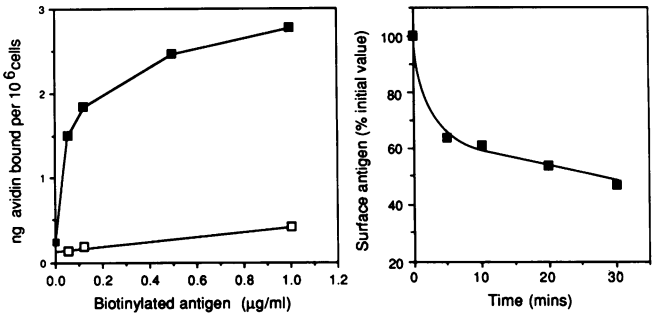

Fig. 4. Binding and endocytosis of biotinylated antigen detected with [^{125}I]avidin. **Left panel.** Cells (clone E8.5) were washed and resuspended in PBS/BSA at 1.3×10^7/ml. Aliquots (0.15 ml) were incubated at 0°C for 2 h with increasing concentrations of biotinylated tetanus toxin in the presence (□) or absence (■) of excess non-biotinylated antigen. After washing the cells free of unbound antigen they were resuspended in 0.15 ml PBS/BSA containing 1.0 μg/ml [^{125}I]avidin and incubated at 0°C for 30 min. The cells were then collected by centrifugation, washed twice with PBS/BSA and bound radioactivity determined. **Right panel.** Cells to which biotinylated antigen had been bound as described above were washed and aliquots (2×10^6 cells) were resuspended in 0.5 ml of PBS/BSA containing 20 mM glucose, incubated at 37°C for the times indicated and then chilled to 0°C. [^{125}I]Avidin binding was determined as described above and expressed as a percentage of the maximal biotinylated antigen-dependent binding.

The initial rate of return of biotinylated antigen to the cell surface corresponded to ~3% of the intracellular antigen per minute over the first 5 min of rewarming and in two separate experiments a total of 30% and 32% of the endocytosed biotinylated antigen could be detected back on the cell surface after a 30-min incubation at 37°C (Figure 5). We suggest that in fact most or all of the intracellular antigen is involved in recycling but is also subject to re-endocytosis during the incubation and so is not, at the end point, scored as being on the cell surface. Instead, we would argue that it becomes redistributed among communicating cell surface and intracellular pools in the same fashion as does [^{125}I]-antigen, initially bound to the cell surface of E8.5 cells; i.e. in the experiment shown in Figure 2 after a 20- to 30-min incubation at 37°C ~55% of the antigen was intracellular and ~35% was on the cell surface (Figure 2). It should be emphasized that recycling of apparently intact antigen is being monitored in these experiments. A minimum period of ~1 h is required before processed antigen/MHC complexes recognizable by T cells appear on the cell surface (Roosnek *et al.*, 1988); moreover, degradation of the radiolabelled antigen is virtually undetectable after the 20 min at 37°C allowed for the initial internalization of biotinylated antigen (Figure 2 and unpublished results).

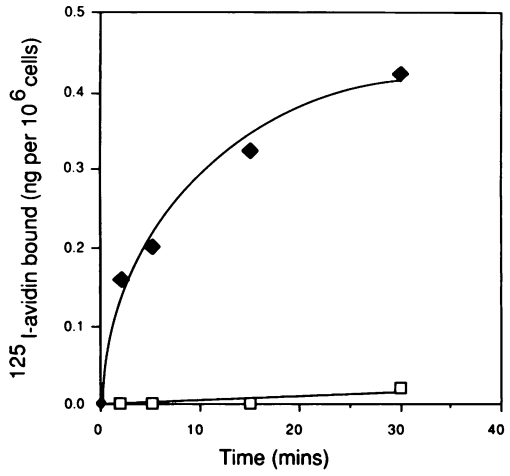

Fig. 5. Exocytosis of previously endocytosed antigen. Cells (E8.5) were incubated with biotinylated antigen (2 μg/ml) at 0°C, washed free of unbound antigen and either held at 0°C (□) or warmed to 37°C for 20 min (◆) in RPMI growth medium. The cells were washed and incubated for 60 min at 0°C in Hanks' salt solution containing 2 mg/ml BSA and 10 μg/ml avidin. Unbound avidin was removed by washing and sites on the bound avidin blocked by a 5-min incubation with 1 μM free biotin. The cells were washed free of biotin and aliquots (2×10^6 cells) rewarmed to 37°C for the indicated times. The time-dependent reappearance of binding sites for avidin was assayed by a subsequent incubation at 0°C with 0.5 μg/ml [^{125}I]avidin as described in Figure 4.

Sustained endocytosis of antigen

If antigen is being recycled to the cell surface at a rate and to the extent indicated by the experiment with biotinylated antigen, then we would strongly predict that this must be balanced by equivalent endocytosis of antigen at time points when net antigen uptake has apparently ceased (Figure 2). A demonstration that antigen endocytosis continues after the first 20 min of incubation would greatly strengthen our conclusion that antigen becomes involved in a steady state of cycling to and from the cell surface. We therefore decided to test this prediction by examining the dynamic state of the cell surface population of antigen molecules at different time points after the initial warm up. We could have used biotinylated antigen, allowed endocytosis to proceed at 37°C and then measured the rate of endocytosis of [^{125}I]avidin bound to the cell surface antigen population. However, we were concerned that the multivalent nature of avidin might influence the rate of endocytosis observed and developed an alternative method of labelling the antigen population present on the cell surface at any given time.

We exploited the fact that the different tetanus-toxin-specific B cell lines recognize different epitopes in the tetanus toxin molecule, and since they secrete soluble IgG into the culture medium of the same specificity as that expressed on their cell surface, we were able to prepare radiolabelled Fabs from the secreted IgGs. As shown in Table I, antigen bound to line E8.5 displayed an epitope recognizable by [^{125}I]Fabs generated from the antibody secreted by line A46 and vice versa. Binding of the [^{125}I]Fabs was blocked by excess unlabelled Fab, demonstrating that binding was specific. The line ALCL-3, which expresses an IgG of unknown specificity, did not bind the anti-tetanus Fabs. Neither of the specific cell lines could bind their own Fabs, a point which serves to confirm that our antigen preparations are truly monovalent (Table I).

Table I. [^{125}I]Fab binding to receptor-bound antigen

Cell	A46 Fab		E8.5 Fab	
	[^{125}I]Fab	[^{125}I]Fab plus excess unlabelled	[^{125}I]Fab	[^{125}I]Fab plus excess unlabelled
ALCL-3	0.2	0.5	0.7	0.6
A46	0.5	0.4	30.5	0.4
E8.5	8.5	0.5	0.9	0.9

B cell lines specific (A46 and E8.5) or non-specific (ALCL-3) for tetanus toxin/toxoid were incubated with 2 μg/ml unlabelled antigen at 0°C for 90 min. After washing the cells free of unbound antigen they were resuspended in PBS/BSA containing 0.5 μg/ml [^{125}I]Fabs in the presence or absence of a 20- to 50-fold excess of unlabelled Fab, and incubated for a further 60 min at 0°C. Radioactivity associated with the cells after washing by centrifugation and resuspension is expressed in fmol/10^6 cells.

To measure antigen endocytosis using this method of labelling, A46 cells were incubated with unlabelled antigen at 0°C and then either held at this temperature or warmed to 37°C for different times and then rechilled to 0°C. Antigen present on the cell surface after these different incubation times was then 'tagged' by incubating at 0°C with the appropriate ^{125}I-labelled Fab. As expected, the cells preincubated at 37°C for 20, 40 or 60 min bound less [^{125}I]Fab than cells held at 0°C, reflecting the loss of cell surface antigen observed in Figure 2. By reincubating the Fab-labelled cells at 37°C we were able to measure the actual rates of endocytosis of antigen on these different cell populations. Our main objective was to compare the rate of Fab (and hence antigen) uptake on cells which had or had not been previously incubated at 37°C to allow antigen redistribution. The initial rate of endocytosis of Fabs bound to cells which had not been previously shifted to 37°C was rapid, corresponding to ~5%/min of the cell-associated [^{125}I]Fab (Figure 6), similar to the initial rate of [^{125}I]antigen endocytosis on this cell line (Figure 2). The initial rate of endocytosis of Fabs bound via antigen to cells preincubated for 20, 40 or 60 min could then be compared to this reference rate. Endocytosis of Fabs on these preincubated cells was also observed and corresponded to ~2%/min of the cell-associated Fab (Figure 6). Although this rate is not as fast as the reference rate, the crucial point to note is that it is some 10 times faster than the net rate of loss of antigen from the cell surface over this 20- to 60-min time period which was constant at ~ 0.2%/min of the antigen initially bound (dotted line in Figure 6); a rate almost 30 times slower than the initial rapid rate of endocytosis (~5%/min on line A46). The endocytosis of Fabs was not simply an artefact of chilling and rewarming of the cells because no stimulation of [^{125}I]antigen endocytosis was observed when cells, engaged in processing a single round of antigen at 37°C, were similarly chilled and rewarmed.

The Fab 'tagging' experiment reveals that antigen remaining on the cell surface after the initial rapid redistribution is still being endocytosed, even at times when the level of intracellular antigen is actually falling (compare Figures 2 and 6). Given that the experiment with biotinylated antigen indicated a rate of endocytosis of antigen of ~3%/min, the sustained rate of endocytosis of cell surface antigen observed here is consistent with the establishment of a steady state where the rates of antigen endocytosis and exocytosis are about the same and the steady decline in cell-associated

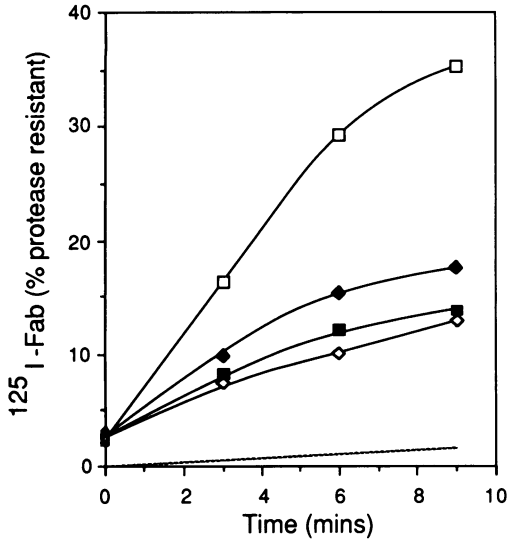

Fig. 6. Fab labelling of antigen demonstrates continued endocytosis. A46 cells were incubated with 2.2 μg/ml unlabelled antigen at 0°C, washed free of unbound antigen and then incubated at 37°C for 0 (□), 20 (◆), 40 (■) and 60 min (◇). The cells were then incubated at 0°C with [^{125}I]anti-tetanus toxin Fab from clone E8.5. After washing off unbound Fab, the cells were reincubated at 37°C for the indicated times, chilled and subjected to pronase digestion. Pronase-resistant Fab is expressed as a percentage of the total cell-associated Fab. The dotted line indicates the expected rate of endocytosis of labelled Fab if it simply reflected the steady-state loss of antigen from the cell surface seen in Figure 2, i.e. after the initial rapid phase of endocytosis.

antigen is due to the release of antigen, mostly degraded, to the medium.

Limited re-use of membrane immunoglobulin in antigen endocytosis

Receptor recycling offers the possibility of capturing more than one antigen molecule per binding site. We suspected that in antigen-specific B cells this might only happen to a limited extent because, unlike other endocytosed ligands such as low-density lipoprotein and asialoglycoproteins, the ligand (antigen) used in these experiments remains tightly bound to its receptor at the mildly acidic pH found in endosomes. We could demonstrate this directly by binding radiolabelled antigen to mIg at 0°C, incubating the cells for various times at 37°C and then precipitating the mIg with an immobilized anti-(human IgG) antibody. After 2 h almost half of the labelled antigen was still bound to the mIgG. It seemed that the slow dissociation of bound ligand might severely limit the re-use of receptors in spite of their ability to recycle.

We decided to examine the capacity of the cells to accumulate antigen directly by incubating tetanus-toxoid-specific B cells in the continuous presence of radiolabelled antigen at 37°C with and without an excess of unlabelled antigen to allow specific uptake to be measured. Cycloheximide was present before and during the incubation to prevent synthesis of new receptors. At different time points the amount of antigen specifically associated with the cells as well as the amount of antigen that had been rendered TCA soluble and returned to the medium was measured. To check that there was still antigen available to the cells at the end of the experiment, we recovered the medium containing the labelled antigen and resuspended fresh cells in it for a further 1 h incubation. The specific accumulation was at least 70% of

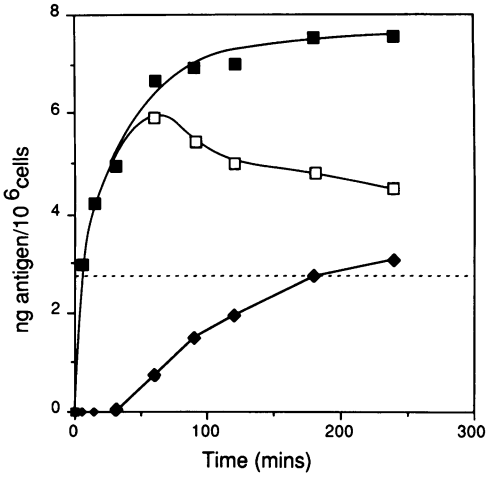

Fig. 7. Continuous incubation with antigen at 37°C. A46 cells were incubated with 2 μg/ml ^{125}I-labelled antigen at 37°C in the presence of 100 μg/ml cycloheximide after first pretreating for 1 h with the same drug. At the times indicated the cells and medium were recovered and assayed, as described in Materials and methods, for specifically associated antigen (□) and antigen rendered TCA soluble (◆) respectively. The sum of these two values (■) and the amount of antigen bound to the cells at 0°C (---) are also indicated.

that shown by the first set of cells providing that cycloheximide had been present during the first incubation. In the absence of cycloheximide the radiolabelled antigen became substantially depleted due to the synthesis and secretion of significant amounts of soluble IgG which then binds to the antigen in the medium (unpublished observations).

The results of these experiments showed that the level of cell-associated antigen initially increased rapidly, reached a maximum after 45–60 min and thereafter gradually declined over a period of 4 h, whilst the amount of TCA-soluble antigen in the medium progressively increased (Figure 7). The kinetics and extent of uptake were very similar to those seen when antigen was prebound at 0°C (Figures 2 and 3). The sum of the cell-associated antigen and that returned to the medium in TCA-soluble form increased rapidly during the first 1 h of incubation but then levelled off. After 4 h it was ~3 times the total binding capacity for antigen at 0°C (Figure 7). However, it should be noted that cells incubated with labelled antigen at 37°C in glucose-free medium containing 20 mM azide and 50 mM 2-deoxyglucose, which completely abolished endocytosis, bound almost twice as much antigen as cells incubated with the same preparation of antigen at 0°C, suggesting that binding to some of the combining sites might be hindered at 0°C. Overall, the capacity of the cells to endocytose and process antigen over a 4-h period at 37°C exceeded their cell surface binding capacity at this temperature by only ~50% (Figure 7), indicating, at best, a very modest re-use of receptors.

Discussion

Until quite recently quantitative biochemical and kinetic analysis of antigen endocytosis has not been possible because, even in lymphoid tissue of hyperimmunized animals, only a small number of B cells specific for the administered antigen are present. Consequently, earlier studies generally employed polyclonal populations of B lymphocytes labelled with anti-(mIg) antibodies and Fab fragments derived there-

from. At 37°C cross-linking of B cell Ig with anti-Ig antibodies leads first to patching in the plane of the membrane and then to the formation of a 'cap' at one end of the cell which is later endocytosed or shed from the cell (Taylor *et al.*, 1971; Loor *et al.*, 1972; Schreiner and Unanue, 1976). Cross-linking of mIg in this fashion may also impart a mitogenic signal (DeFranco *et al.*, 1982), which has led to the notion that cross-linking of mIg by antigen might be a crucial event leading to B cell proliferation and differentiation into plasma cells. Fab fragments against mIg failed to trigger either capping or mitogenesis, although recent work has shown that monovalent Fabs against mIg can in fact be endocytosed (Goud and Antoine, 1984; Metezeau *et al.*, 1984) and can indeed be presented as antigens to anti-Ig-specific T cells without any requirement for mIg cross-linking (Tony *et al.*, 1985). As we and others have discussed elsewhere (Watts and Howard, 1986; Abbas, 1988), the 'capping' image may be more appropriate for thymus (T cell)-independent B cell activation by antigen than for T-cell-dependent activation.

Whereas phagocytosis of cross-linked mIg occurs after a 5-min lag (required to form the cap; Schreiner and Unanue, 1976), endocytosis of monovalent antigen proceeded immediately on warming the cells to 37°C (Figures 2 and 3). The observed kinetics ($t_{1/2}$ = 8.5 min) are ~2-fold slower than those seen for other receptor-bound ligands, e.g. transferrin (Ciechanover *et al.*, 1983; Hopkins and Trowbridge, 1983), LDL (Brown and Goldstein, 1976) and EGF (Carpenter and Cohen, 1976); these ligands being endocytosed, with typical half-times of 3–5 min, through clathrin-coated pits (Pearse, 1987). Our preliminary results suggest that this is also the route of entry for antigen bound to mIgG.

Why has the capacity of uncrosslinked mIg to be rapidly endocytosed not been revealed before? Two factors may have contributed to this. Firstly, the Ig class to which antigen or anti-mIg Fabs are bound may influence the rate of endocytosis. The cells used here were isolated from human peripheral blood, express mIgG and correspond most closely to memory B cells, whereas B lymphocytes isolated from lymphoid tissue mostly express mIgM and/or mIgD. It will be interesting to see if antigen bound specifically to mIgM and mIgD can be as efficiently endocytosed as that bound to mIgGs. Membrane IgG displays a cytoplasmic domain of 28 amino acid residues which may allow a more efficient interaction with the endocytic apparatus than the three cytoplasmic residues found on mIgMs and mIgDs (Rogers *et al.*, 1980; Tyler *et al.*, 1982). Conceivably, the ability to endocytose rapidly monovalent antigen is an important aspect of memory cell function for which the cytoplasmic domain of mIgG is best suited.

We should also consider the possibility that Fab/mIg complexes are not as efficiently endocytosed as antigen/mIg complexes. Although the separate domains which make up an immunoglobulin molecule do not appear to be well adapted for this purpose, it is none the less conceivable that binding of antigen to the combining site induces long-range conformational changes which might, in the case of mIg, be detected by the endocytic apparatus. However, binding of anti-mIg Fabs to mIg regions other than the antigen-binding site may not faithfully mimic the binding of antigen, and so may not be as efficiently endocytosed.

Intrinsic to the structure of several recycling receptors is the ability to bind their ligands tightly at neutral pH but only weakly at mildly acidic pH. Endosomes maintain a pH of 5–6 which allows such receptors to deliver their ligands and be reused without being exposed to the potentially destructive environment of the lysosome (Mellman *et al.*, 1986). Although there might be advantages in uncoupling the interaction between the antigen and antibody at mildly acidic pH to allow a single mIgG molecule to be reused many times, this is probably not compatible, for most antibody/antigen combinations, with the requirement for high affinity at neutral pH. Such a restriction on the re-use of receptors can explain the observations made in this study that the capacity of the cells to accumulate antigen was limited when protein synthesis was blocked (Figure 7). In this respect it is interesting to note that fibroblasts expressing a mutant LDL receptor, unable to release its ligand at low pH, showed a limited capacity to endocytose and degrade LDL compared to cells expressing wild-type receptors (Davis *et al.*, 1987). The mIg receptor for antigen also resembles the transferrin receptor (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983) in that ligand and receptor are recycled but, unlike transferrin, antigen remains bound at the cell surface and is subsequently re-endocytosed (Figure 6).

How is proteolytic processing and presentation of antigen achieved? Although at present there is no definitive evidence, a plausible scheme for transport of material through the endocytic pathway is the 'maturation' pathway (Helenius *et al.*, 1983) whereby material reaches lysosomes essentially by default, in the sense that it fails to be recycled from a maturing endosome or one about to fuse with a lysosome. Although mIg can be recycled to the cell surface together with its bound antigen, proteolytic breakdown of the antigen does none the less eventually occur. Since after prolonged chase times much of the radiolabel was found in the medium in TCA-soluble form, most of the antigen must ultimately reach a protease-rich compartment. This could mean that recycling from peripheral endosomes was < 100% efficient, resulting in a slow transfer of mIg-bound antigen to endosomes containing proteases (Diment and Stahl, 1985; Roederer *et al.*, 1987), or that such compartments might be part of the major recycling pathway. Passage through such compartments might offer a controlled way of introducing cleavages into the antigen molecule without completely destroying it in lysosomes. Our preliminary results indicate that proteolytic processing begins while the antigen is still bound to the mIg, in which case those parts of the antigen molecule not involved in binding to the mIg would probably be most accessible and might therefore be preferentially cleaved; in other words, the epitope through which antigen is bound might influence the processing pathway, the fragments subsequently presented and so the T cell clones which can form collaborations with a particular B cell. Some evidence for such preferential T cell/B cell pairings exists (Manca *et al.*, 1985; Lanzavecchia, 1986; Ozaki and Berzofsky, 1987) and might be explored most readily with B cell clones recognizing non-overlapping epitopes in the same antigen. In this regard complete sequence information is now available for tetanus toxin (Eisel *et al.*, 1986; Fairweather and Lyness, 1986) and the epitopes recognized by the B and T cell lines available are currently being mapped.

The crucial question of when and how processed antigen becomes associated with the Class II molecules that eventually present it on the cell surface remains to be addressed. We favour the idea that MHC Class II and processed antigen, possibly still bound to the mIg, must be brought together

in some non-lysosomal intracellular compartment. Whether antigen fragments are released and become subsequently bound to Class II or whether some kind of concerted transfer occurs without complete dissociation from the membrane surface is a question for the future. Processed antigen, at least in its immunologically relevant form, has not yet been observed experimentally. Endocytosis of antigen by B cells is shown here to be rapid and efficient, enabling concentrations of antigen as low as 10^{-12} M to activate specific T cells (Lanzavecchia, 1985). These factors should facilitate the elucidation of the pathway of MHC-restricted antigen presentation at the biochemical and morphological level.

Materials and methods

Cells and antigens

Human B cell lines specific for tetanus toxoid were generously provided by Dr A.Lanzavecchia, Basel Institute for Immunology, Basel, Switzerland. Their isolation and capacity to present this antigen to specific T cells has been previously described (Lanzavecchia, 1985). The cells were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM pyruvate, non-essential amino acids and antibiotics (Flow Labs).

Affinity-purified tetanus toxoid was a gift from Wellcome Biotech, Beckenham, UK. Tetanus toxin was generously provided by Dr S.van Heyningen, University of Edinburgh and by Dr N.Fairweather, Wellcome Biotech. Both toxin and toxoid were further purified by gel filtration chromatography on a 2 × 89 cm column of Sephacryl S-300 (Pharmacia) equilibrated in 1.0 M NaCl, 0.1 M Tris−Cl, pH 8.0. Antigen chromatographing with an apparent mol. wt of 150 000 was pooled, dialysed against 10 mM sodium phosphate, pH 7.2, and lyophilized. Tetanus toxin C fragment was purchased from Calbiochem and used without further purification.

Monovalent antigen (5 μg) was radiolabelled by the Iodogen method (Fraker and Speck, 1978) in 20 μl 0.1 M phosphate, pH 7.2, to which 0.5 mCi ^{125}I (Amersham) was added. After a 15-min incubation at 0°C the reaction was quenched by the addition of 75 μl 50 mM KI and the mixture applied to a 6 ml column of Sephadex G-50 equilibrated in phosphate-buffered saline (PBS). Fractions (~300 μl) were collected and those containing radioiodinated antigen were pooled, made 1 mg/ml in bovine serum albumin (BSA) and dialysed against PBS at 4°C. The specific activity of the antigen was ~2 × 10^4 c.p.m./ng. Anti-tetanus toxin Fabs (see below) and avidin (Vector Laboratories) were iodinated in the same way to a similar specific activity. Protein concentrations were assayed by the method of Bradford (1976), using BSA as a standard.

Binding and endocytosis of antigen

Cells were removed from culture, collected by centrifugation (4 min at 500 *g*), washed in ice-cold PBS containing 5 mg/ml BSA and resuspended at a density of 2 × 10^7 cells/ml in PBS/BSA. Radioiodinated antigen was added in the presence or absence of a 100-fold excess of unlabelled tetanus toxoid to establish the extent of specific binding, which was usually between 90 and 98%. After incubation at 0°C for 60−90 min the cells were then collected by centrifugation, washed to remove unbound antigen and resuspended to a density of 4 × 10^6 cells/ml in growth medium supplemented with 20 mM Hepes, pH 7.2. Aliquots of the cells were incubated at 37°C for different periods of time, rechilled to 0°C and the cells collected by centrifugation. Two methods were used to differentiate between cell surface and intracellular antigen. Cells were either incubated with 1.5 mg/ml pronase (Sigma Protease XIV) in PBS at 0°C for 1 h or incubated for 15 min in 20 mM HCl, 150 mM NaCl (pH 1.7) at 0°C. The digested or acid-washed cells were pelleted and radioactivity found in the supernatant assumed to be cell surface antigen. Both methods removed >90% of antigen specifically bound to mIg at 0°C. All samples were counted on an LKB 1261 Multigamma counter.

When antigen was supplied continuously at 37°C the cells were first preincubated in complete growth medium containing 100 μg/ml cycloheximide for 1 h. ^{125}I-Labelled antigen (2 μg/ml) was added to cells resuspended at a density of 5 × 10^6/ml in medium containing cycloheximide with or without an excess of unlabelled tetanus toxoid. At different time points the cells were collected and cell-associated antigen assayed as described above. Trichloroacetic acid was added to the medium containing radiolabelled antigen to a final concentration of 10% (w/v) and TCA-soluble radioactivity assayed according to the method of Goldstein and Brown (1974).

Preparation of Fab fragments

The medium used for growing the cells was retained after removing the cells by centrifugation and stored in the presence of 5 mM sodium azide at 4°C. IgG present in the media was purified by chromatography on Protein A Sepharose with a yield of 3−5 μg/ml medium. Fab fragments were generated by cleavage with immobilized papain (Sigma) at 37°C and purified away from intact antibody and Fc fragments by rechromatography on Protein A Sepharose.

Biotinylation of tetanus toxin

Tetanus toxin (1 mg/ml) was biotinylated using a biotin *N*-hydroxy-succinimide ester (long arm) according to the instructions supplied by the supplier (Vector Labs). A 10-fold molar excess of the biotin ester was added to the toxin at 23°C. After stirring at 23°C for 1 h the antigen was dialysed against PBS at 0°C. Binding to tetanus-specific B cells was performed as described above except that after washing the cells free of unbound biotinylated antigen, bound material was detected with [^{125}I]avidin (see figure legends for details).

Acknowledgements

We are most grateful to Antonio Lanzavecchia, Basel Institute for Immunology, for providing the cell lines used in this work and for valuable discussions. We are also indebted to Simon van Heyningen, Neil Fairweather and Wellcome Biotech for generous gifts of tetanus toxin and tetanus toxoid. We thank our colleagues for valuable discussion and criticism of the manuscript. This work was supported by a Medical Research Council Project Grant and by the Nuffield Foundation.

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Received on January 13, 1988; revised on April 11, 1988