Internalization and catabolism of radiolabelled antibodies to the MHC class-II invariant chain by B-cell lymphomas

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The fate of antibody (Ab) LL1, which reacts with the invariant chain (Ii) subunit of the immature MHC class-II antigen (CD74) after binding to the surface of B-cell lymphomas was investigated. This Ab was internalized and catabolized very rapidly, much faster than other Abs that are considered to be rapidly internalized, such as CD19, CD22 and anti-(transferrin receptor). Such internalization did not depend on Ab cross-linking. The capacity of this uptake process was determined in long-term

experiments by increasing the Ab concentration: in 1 day, approx. 8×10^6 Ab molecules per cell were catabolized. This analysis was facilitated by the use of radiolabels that are trapped within cells after catabolism of the Abs to which they were conjugated. If the Ab is a reliable marker for the Ii antigen, which is likely, we can conclude that Ii directed to the cell surface appears to be sufficient, indeed more than sufficient, to account for the cell content of mature class-II molecules.

INTRODUCTION

The binding of antigenic peptides to the MHC class-II antigens is a key process in the immune response. Class-II antigens are synthesized initially with an extra polypeptide chain, the invariant chain (Ii), which obstructs peptide binding. At an intracellular site, this chain is removed as a result of proteolytic cleavage, and peptide binding occurs. Mature class-II molecules bearing peptides are then transported to the cell surface, where they present the peptide to T-lymphocytes. The mechanism by which immature class-II molecules and antigenic peptides, originating from endocytosed proteins, are gathered together has been extensively investigated [1–7]. It is useful to consider that peptide delivery to class-II molecules can occur by either of two basic pathways. The first is a receptor-mediated pathway that handles antigens binding specifically to surface immunoglobulin on B-cells [1,7], or antigens binding via antibodies (Abs) to the Fc receptors on macrophage-like cells [8]. The second is a non-specific pathway that handles other antigenic molecules present in the environment of the antigen-presenting cell.

Currently, the most widely accepted model is that most immature class-II molecules do not reach the cell surface before they have bound peptide and been converted into the mature form. However, the presence of some immature class-II molecules on the cell surface has been demonstrated [9–12]. The amount detected is low, and this pathway has been considered to be minor, perhaps resulting from the mis-sorting of a small fraction of the molecules [2,4,6]. However, low steady-state levels are not incompatible with the possibility that most or all immature class-II molecules briefly appear on the cell surface. Koch et al. [10] first presented evidence that cell surface Ii is rapidly internalized, based on experiments with brefeldin. Roche et al. [12] demonstrated by various experimental approaches that cell surface Ii is internalized rapidly, by a constitutive process, and suggested that large amounts of immature class-II molecules

were directed to the cell surface (approx. 4×10^6 molecules/cell per day). However, this calculation was based on a 20 min experiment. To investigate this question further, we have focused on the catabolism of bound anti-Ii Abs, which was not previously analysed, over periods of up to 3 days. Also, we have utilized residualizing radiolabels [13,14], which are trapped within the cells, probably within lysosomes, after catabolism of the Ab to which they were conjugated. Since the internalization and catabolism of anti-Ii Abs by B-cell lymphomas is so rapid, it is difficult to follow with a conventional ^{125}I label, which is rapidly released from the cell as iodotyrosine after Ab catabolism [14,15]. Two residualizing labels were used, namely ^{125}I -dilactitol-tyramine (^{125}I -DLT) and the ^{111}In chelate of benzyl-diethylenetriaminepenta-acetic acid (DTPA). The data obtained confirm the constitutive internalization of cell surface Ii in large amounts, with approximately 10^7 molecules/cell per day being internalized. This high value suggests that it may be useful to reconsider the possibility that all immature class-II molecules briefly appear on the cell surface.

MATERIALS AND METHODS

Cell lines and antibodies

The B-cell lymphomas used were: Raji, obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.); RL [16], obtained from Dr. John Gribben (Harvard Medical School, Boston, MA, U.S.A.); and JY [17], obtained from Dr. Eric Long (National Institutes of Health, Bethesda, MD, U.S.A.). The mutant B-cell line .174, which expresses Ii but not class-II α - or β -chains, has previously been described [12]. Cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 12.5% fetal bovine serum, penicillin (10 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), glutamine (2 mM) and sodium pyruvate (1 mM) (Gibco). Abs LL1, an IgG₁,

Abbreviations used: DLT, dilactitol-tyramine; DTPA, diethylenetriaminepenta-acetic acid; EGF, epidermal growth factor; Ab, antibody; Ii, invariant chain.

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and LL2, an IgG_{2a} reacting with CD22, have previously been described [18,19] (originally named EPB-1 and EPB-2 respectively). LL1 was previously described as reacting with a class-II-like molecule [18], but, as demonstrated here, it reacts with a cell-surface-expressed epitope of the Ii subunit. Other Abs to Ii (CD74) were LN2 [11], provided by Dr. Alan Epstein (University of Southern California Medical Center, Los Angeles, CA, U.S.A.), and BU45 [11] and POP-I4.3 [20], provided by Dr. Eric Long. An anti-peptide Ab to the Ii cytoplasmic domain, Pin1.1, has been described previously [21]. Abs to the mature class-II antigen were DA6.147, anti-DR α [12] and Lym-1 [22], which was provided by Dr. Gerald DeNardo (University of California-Davis, Sacramento, CA, U.S.A.). Ab 5E9, an IgG₁ reactive with the transferrin receptor, was produced by hybridoma cells obtained from ATCC. Anti-CD19 was purchased from Biosource (Camarillo, CA, U.S.A.). If required, IgG Abs were purified from ascites fluid by Protein A affinity chromatography by methods that have been described [14]. The Fab' fragment of LL1 was generated and characterized by standard procedures [23], and was provided by Dr. Ken Chang (Immunomedics).

Antibody radiolabelling

Procedures for labelling Abs with ¹²⁵I by conventional methods (chloramine T), ¹²⁵I-DLT and ¹¹¹In, using isothiocyanate-benzyl-DTPA, have been described previously [14]. The specific radioactivities were 10–20 mCi/mg for conventional iodine, approx. 1 mCi/mg for DLT and 5–20 mCi/mg for indium. Conjugated Abs were analysed by gel-filtration HPLC on a Bio-Sil SEC-250 column (Bio-Rad, Hercules, CA, U.S.A.), and more than 95 % of the radioactivity migrated as the expected IgG or fragment. In some experiments, unlabelled Ab was added to obtain the desired specific radioactivity.

Standard Ab-processing experiments

All incubations were performed in tissue culture medium at 37 °C, except where noted. Cells (10⁷) were centrifuged, washed once, then incubated in 1 ml containing 10⁷ c.p.m. of Ab, for 1 h. To ensure the specificity of Ab binding, controls were set up in every experiment, containing 10-fold smaller samples (cells, Ab and medium), with and without excess unlabelled Ab (50 μ l at 50–250 μ g/ml). In all experiments, at least 90 % of the bound radioactivity was specifically inhibited by the unlabelled Ab. After the binding incubation, cells were washed four times with 5 ml of tissue culture medium by centrifugation. The specificity controls were counted, and the large portion was suspended in 30 ml, then dispensed in a 24-well plate (Falcon, VWR Scientific, Piscataway, NJ, U.S.A.), with 1.5 ml/well. Samples of 1.5 ml were saved for radioactivity determination, which was the 'initially bound' c.p.m. The plate was incubated in a humid incubator containing 5 % CO₂, and at 3, 21, 45 and 69 h, the cells and supernatant were collected as follows. Cells were suspended by repeated pipetting, and transferred to conical tubes. The wells and pipette were rinsed with 1 ml of tissue culture medium, which was added to the initial cell suspension collected. The tube was centrifuged for 10 min at 940 *g* (2000 rev./min), and 1 ml of supernatant was carefully collected (40 % of the total supernatant). The supernatant was counted for radioactivity, 1 mg of bovine IgG was added as carrier protein, and the protein was precipitated. For experiments using iodine alone, 5 ml of cold 10 % (w/v) trichloroacetic acid was used; for experiments with indium, 5 ml of methanol was used, because trichloroacetic acid caused release of indium from the chelator. After incubation for 30 min at 4 °C and centrifugation for 15 min at 4300 *g* (6000 rev./min), the supernatant was discarded, and the precipitated

protein was counted for radioactivity. Radioactivity not precipitated by trichloroacetic acid or methanol was considered degraded, and precipitated radioactivity was considered intact. We realized that large Ab fragments would also be precipitated, but such partially degraded Ab is unlikely to appear in the supernatant [15,25]; this question is of no concern in most of these experiments because there was very little release of 'intact' LL1 into the supernatant. The original cell pellet was washed once with 5 ml of tissue culture medium, and the pellet counted for radioactivity. Radioactivity is expressed as a percentage of that initially bound. To inhibit Ab catabolism, NH₄Cl at 10 mM or leupeptin (L2884, Sigma Chemicals, St. Louis, MO, U.S.A.) at 0.1 mM was included in the medium in which cells were plated, after coating with Ab. In some experiments, the medium used for the Ab incubation, and for pretreating cells for 30 min before the Ab incubation, contained 10 mM NaN₃, 5 mM 2-deoxyglucose (Sigma) and 5.0 % dialysed fetal bovine serum (Gibco) and was glucose-free. Competitive binding experiments were performed similarly to the specificity controls described above, except that different unlabelled Abs were used.

Prolonged Ab incubations

Wells of 24-well plates contained 1.5 ml of medium, 5 $\times$ 10⁶ cells and 3 $\times$ 10⁷ c.p.m. The Ab protein concentration was varied systematically, as described in the text. At 4, 24, 48 and 72 h, wells were harvested as described above, except that only 0.25 ml of the initial supernatant was collected (10 % of the total), so that the radioactivity would not be too high for accurate counting. In experiments with double radiolabels, c.p.m. were corrected for cross-over. The radioactivity 'handled' is defined as the cell-bound radioactivity plus the degraded radioactivity in the supernatant, and indicates the total Ab bound and processed by the cells. In calculating the Ab molecules handled per cell, a correction was made for the percentage of radioactivity in the Ab stock solution not precipitated by trichloroacetic acid or methanol (always less than 20 %). In addition, the possible deterioration of the labelled Ab incubated in tissue culture medium at 37 °C was monitored: the ¹¹¹In-label became detached from intact Ab at a rate of approximately 1.4 % per day, which was insignificant in most experiments. Binding specificity in these 'prolonged Ab incubation' experiments could not be readily determined by inhibition with excess unlabelled Ab, since under these conditions even a large amount of unlabelled Ab (up to 50 μ g/ml, which required 0.75 mg/experiment) was unable to totally block specific Ab uptake, partly because of the very high capacity for uptake. Therefore non-specific uptake was determined by testing a non-reactive Ab labelled with ¹¹¹In and incubated under the same conditions. Non-specific Ab uptake was very low: the cell-bound radioactivity was only 0.1 % at 24 h and 0.4–0.5 % at 48 h. The radioactivity degraded by the cells and released into the medium (corrected for the spontaneous deterioration of the Ab) was also very low, at a rate of approximately 0.5–1.5 % per day.

Other methods

Fluorescent staining of the surface of viable cells and FACS analysis utilized a fluorescein-conjugated goat anti-mouse IgG, as previously described [25]. Negative control cells were treated with a non-reactive Ab of the same subclass. Methods of antigen analysis by immunoprecipitation have also been described [25]. Briefly, 3.0 $\times$ 10⁷ JY cells were labelled with 0.5 mCi of [³⁵S]methionine for 4 h, washed, and extracted with Triton X-100. After immunoprecipitation with Protein A-Sepharose, precipitated proteins were analysed by two-dimensional

electrophoresis (non-equilibrium pH-gradient electrophoresis followed by reducing SDS/PAGE).

RESULTS

Characterization of the specificity of Ab LL1

It was originally suggested, on the basis of immunofluorescence with normal blood cells and a panel of tumour cell lines, immunohistology with normal tissues, and Western immunoblots of cell extracts [18], that LL1 reacts with MHC class-II-like

Table 1 Competitive binding assay with ^{125}I -LL1 binding to Raji cells

Inhibitor was present at a concentration of 0, 10, 50 or 250 $\mu\text{g}/\text{ml}$. Values shown are means $\pm$ S.D. from duplicate determinations. Similar results were obtained in repeated experiments.

Inhibitor	$10^{-3} \times \text{Radioactivity (c.p.m.)}$			
	0	10	50	250
Medium	86.8 $\pm$ 0.7	—	—	—
Negative IgG ₁	—	—	—	94.5 $\pm$ 1.8
LL1	—	20.6 $\pm$ 0.7	6.6 $\pm$ 0.2	2.6 $\pm$ 0.1
LN2	—	66.6 $\pm$ 1.5	55.9 $\pm$ 2.4	31.3 $\pm$ 0.4

Figure 1 Immunoprecipitation with Ab LL1 compared with known Abs to Ii and DR α

Cells were labelled with [^{35}S]methionine for 4 h, and immunoprecipitates were analysed by two-dimensional electrophoresis and autoradiography of the dried gels. The Abs used were: (A) DA6.147 (anti-DR α); (B) LL1; (C) Pin1.1 (anti-Ii cytoplasmic domain). The positions of the α , β and Ii chains are indicated. The LL1 immunoprecipitate looks identical with the known anti-Ii immunoprecipitate.

Figure 2 The fate of ^{125}I -labelled Abs bound to the surface of Raji cells

After a binding incubation, unbound Ab was washed away, and the fate of the bound Ab followed for up to 3 days. In all cases, circles show cell-bound radioactivity, squares show degraded radioactivity released into the supernatant, and triangles show intact Ab released into the supernatant. (A) Solid symbols, LL1 with a conventional ^{125}I label; open symbols, LL1 labelled with ^{125}I -DLT. Approx. 70% of the conventional label was degraded and released from the cells within 3 h, but this was markedly inhibited with the residualizing label DLT. (B) Early time points of LL1 processing, showing that most of the catabolism was accomplished within 1 h. (C) The fate of three other Abs, shown for comparison with LL1. Solid symbols, anti-CD22 LL2; open symbols with dashed lines, anti-CD19; small open symbols with dot-dashed lines, anti-mature class-II, Lym-1. (D) The inhibition of LL1 catabolism with NH_4Cl at 10 mM was included in the medium, which markedly inhibited catabolism. Results shown are means of duplicates. Standard deviations are omitted for clarity, but were always less than 3% of the total bound radioactivity. In all experiments, more than 90% of the Ab binding was antigen-specific, as shown by inhibition with excess unlabelled Ab in control tubes. Each experiment was repeated at least twice with similar results.

molecules. The immunoblot data are consistent with Ii reactivity, with a doublet at 35 and 28 kDa and a minor band at 45 kDa [18]. The finding that the Ab was internalized and catabolized very rapidly suggested that it might react with Ii, as similar results with the Ab BU45 were obtained by Roche et al. [12]. Therefore LL1 was compared with known anti-CD74 Abs by three methods. First, competitive binding experiments were performed with three Abs to Ii, which identify two epitopes [11]. LL1 binding was inhibited by LN2, but not by BU45 or POP.14.3, which is consistent with previous epitope analysis. As shown in Table 1, LN2 inhibition was relatively weak, though clear, and LN2 was approximately 25-fold less active than LL1 at inhibiting the binding of radiolabelled LL1. In the reciprocal experiment, radiolabelled LN2 bound weakly to the cell surface, with only approx. 3000 c.p.m. specifically bound, but this binding was completely inhibited by LL1 or LN2, and not by other Abs. The difference in avidity between these two Abs is indicated by the difference in the amount of radioactivity specifically bound: with a total of 500000 c.p.m./tube, containing 10^6 cells, approx. 80000 c.p.m. of LL1 specifically bound, compared with only 3000 c.p.m. of LN2. Such results are consistent with previous data indicating that LN2 reacts quite weakly with the cell surface; in fact, it was originally described as not reacting with the cell surface [11]. In contrast, LL1 reacts strongly with the cell surface as assessed by all assays performed, including immunofluorescence (R. Stein, personal communication). Thus, of the anti-Ii Abs tested, LL1 appears to be unique in that it reacts with the LN2 epitope, yet binds strongly to the cell surface. The number of specific Ab-binding sites per cell was determined

Figure 3 Effect of incubation temperature on the processing of LL1 bound to Raji cells

The 1 h Ab incubation was at either 37 °C (standard conditions) or 0 °C. (A) Total radioactivity bound, which is nearly 3-fold higher at 37 °C than at 0 °C. (B) Subsequent processing of the bound Ab, after 37 °C (filled symbols) or 0 °C (open symbols) Ab incubations. Circles, cell-bound radioactivity; squares, degraded radioactivity in the supernatant; triangles, intact Ab in the supernatant. Values shown are means of duplicates; standard deviations were all less than 3% of the total bound radioactivity. A repeated experiment had similar results.

by incubating Raji cells in serial dilutions of LL1, to obtain Ab excess. At saturation (2.5 µg/ml) approx. 4.8×10^4 Ab molecules/cell were specifically bound at 4 °C (results not shown).

The specificity of LL1 for Ii was also demonstrated by immunoprecipitation, in a comparison with Pin1.1, an Ab to the intracellular domain of Ii, and DA6.147, an Ab to the class-II DR α chain (Figure 1). The [³⁵S]methionine-labelled proteins precipitated by LL1 looked identical with those precipitated by the known anti-Ii Ab, and characteristically different from those precipitated by the anti-(DR α chain) Ab, in having more Ii relative to α - and β -chain. This difference can be attributed to the fact that the Ii Abs precipitate free endoplasmic-reticulum-retained Ii [1] as well as $\alpha\beta$ Ii complexes. We note that the 4 h labelling period is not long enough to allow significant levels of mature $\alpha\beta$ complexes to be produced. Furthermore, by immunofluorescence, LL1 reacted strongly with the .174 cell line, which lacks the class-II α - and β -chains but expresses Ii; LL1 reacted as strongly as the known anti-Ii Ab, BU45. We conclude that LL1 is an anti-CD74 Ab.

Rapid catabolism of LL1 bound to B-cell lymphomas

¹²⁵I-labelled LL1 bound to either Raji or RL was catabolized rapidly. As shown in Figures 2(A) and 3(B), nearly all of the bound radioactivity was catabolized and released into the supernatant within 3 h. This is much faster than with other Abs that are considered to be rapidly internalized, such as anti-CD22 and anti-CD19, which are shown in Figure 2(C) for comparison. These other Abs were substantially catabolized, but this was barely detectable at 3 h, and required 2 days or more for completion. Figure 2(C) also shows results with Ab Lym-1, which reacts with the mature class-II molecule, and which was catabolized very slightly over 2 days. We note that with both anti-CD19 and Lym-1, a large fraction of the bound Ab dissociated intact from the cell surface, perhaps indicating low avidity. Such dissociation would tend to reduce the amount of

Ab that will be catabolized, since dissociation and internalization can be considered to be competing processes. For very rapidly internalized Abs such as LL1, dissociation may be too slow to have an effect. Ab 5E9 binding to the transferrin receptor was also tested, and was processed quite similarly to anti-CD22 (results not shown). Catabolism and release of bound LL1 was inhibited by either NH₄Cl or leupeptin, which inhibit lysosomal enzymes by different mechanisms [24]. NH₄Cl was quite effective, as shown in Figure 2(D). Leupeptin was considerably less effective, but did significantly inhibit Ab catabolism at early time points. Very similar results were obtained with an Fab' fragment of LL1, demonstrating that the rapid catabolism did not require bivalent Ab binding. With the Fab' fragment, more than 80 % of the bound radioactivity was catabolized and released within 4 h, and most of this had been released by 1 h.

To support further the conclusion that LL1 was catabolized intracellularly, experiments were performed with ¹²⁵I-DLT, a residualizing radiolabel which is known to be trapped within the cell after catabolism of the Ab to which it had been conjugated [15]. Such radiolabels were in fact retained by the cells much longer than a conventional iodine label (Figure 2A), which is excreted by the cell in the form of iodotyrosine after Ab catabolism [15,24].

Effect of low temperature (0–4 °C) on LL1 processing

Since we are interested in events occurring under physiological conditions, our experiments are routinely performed at 37 °C. However, experiments of this type are often performed at 0 °C. It is widely assumed that the low temperature 'immobilizes' the cells temporarily, therefore effectively allowing synchronization of Ab binding. That is, the time required for Ab binding, and washing away unbound Ab, is considered to be effectively 0 min, since these procedures are performed at 0 °C. Figure 3 demonstrates, however, that the change in temperature significantly affects the results obtained. Using a low-temperature Ab incubation, the subsequent release of intact Ab is markedly increased (by approximately 3-fold to 27 %), the catabolism of bound Ab is markedly decreased, and the retention of Ab by the cells is markedly increased (Figure 3B). Also, the total binding of Ab is much higher at 37 °C than at 0–4 °C, as shown in Figure 3(A), which would be expected as a result of the rapid constitutive internalization of Ii. The mechanism of the low-temperature effect has not been determined, although we noted a tendency for increased cell debris after 0 °C treatment, which would be consistent with the idea that membrane vesicles are shed, as described by Kaplan and Keogh with a different cell line [25]. We describe in the Discussion other data suggesting that low temperature may have significant effects on experiments of this type. Our conclusion is that, although low-temperature binding incubations are advantageous for many experimental purposes, the temperature of Ab incubations can have a significant impact on experiments of the type performed here, and must be taken into consideration.

Metabolic inhibitors were also tested as an alternative approach to inhibit internalization and catabolism during the Ab-binding incubation. NaN₃ alone, at 10 mM, only partially inhibited Ab catabolism. Azide combined with 5 mM 2-deoxyglucose did effectively inhibit catabolism; however, similar to the effect of a 0 °C binding incubation, the cells never fully recovered from the 1.5 h drug treatment, in that the percentage of radioactivity remaining on the cells after 21 h was still 31 %; in contrast, control cells retained only 11 % at 3 h, and 5 % at 21 h.

Figure 4 Processing of LL1 labelled with either ^{111}In or conventional ^{125}I by Raji cells, in a prolonged Ab incubation

Cells were incubated with a mixture of both radiolabelled Abs for the entire incubation period. Circles, cell-bound radioactivity; squares, degraded radioactivity in the supernatant; triangles, total radioactivity 'handled' (equal to the sum of the first two parameters). Indium (filled symbols) accumulates to high levels within cells, whereas iodine (open symbols) does not, but instead appears degraded in the supernatant. The Ab concentration was $0.31 \mu\text{g/ml}$. Values shown are means of duplicates, and standard deviations were all less than 3% of total radioactivity. In control experiments with a non-reactive ^{111}In -Ab, only 0.5% was cell-bound at 48 h, so the great majority of uptake was antigen-specific. Results shown are representative of three experiments.

Attempted elution of bound LL1 Ab at low pH

An important question is whether the processing of bound LL1 reflects the processing of the Ii antigen recognized. If the Ab-antigen complex dissociates at the low pH encountered in intracellular compartments, this may not be the case. To investigate this point, LL1 was bound to the surface of Raji cells at 4°C (to ensure that all bound Ab remained on the cell surface). After washing of the cells at 4°C , they were resuspended in low-pH buffers at room temperature or at 37°C for 5 min, and elution of the bound Ab was monitored. The buffers tested were 0.02 M sodium acetate buffer, 0.13 M NaCl, 0.9 mM CaCl_2 , 0.5 mM MgCl_2 , pH 4.0 or 5.0, to test the pH range encountered in acidic endosomal vesicles. No elution was detected under these conditions.

Ab uptake during prolonged Ab incubation with residualizing radiolabels

Data of Roche et al. [12] suggested that Ii uptake from the cell surface is a constitutive process. Based on short-term (20-min) experiments, they calculated that 4.3×10^6 Ab molecules/cell per day would be taken up. To demonstrate this directly, experiments were performed with prolonged Ab incubations. In these experiments, cells were incubated in the presence of Ab for up to 3 days. In such experiments, we would expect a residualizing label to accumulate within the cells, although some excretion of residualizing catabolic products does occur [14]; in contrast, a conventional iodine label would not accumulate within the cell, but should accumulate in the supernatant in degraded form. Ab catabolism by the cells can potentially be demonstrated with either type of radiolabel, but the results with the residualizing label can be considered to be somewhat clearer. In our initial experiments, a double label was utilized, with ^{125}I - and ^{111}In -labelled Abs mixed in the same wells. The results, shown in Figure 4, demonstrate a dramatic difference between the two

radiolabels, as expected. The ^{111}In accumulated gradually within cells to a very high level, up to 32.2% of the total radioactivity per well at 24 h, whereas the ^{125}I never accumulated to more than 1.9% of the total iodine radioactivity. Thus, with this Ab bearing a residualizing label, the radioactivity that binds in a typical 1 h incubation provides no indication of the massive amount of Ab that would be taken up during a prolonged Ab incubation. Similar 3-day experiments with an $^{111}\text{In}/^{125}\text{I}$ double label were performed with two other Ab-target cell combinations: anti-CD22 (LL2) on Raji and RS7 on Calu-3 lung carcinoma cells. In both cases, the Abs are considered to be rapidly internalized and degraded, although of course this is much slower than with LL1. In these studies, the indium label was more effective than the iodine label, but the difference was much less than with LL1, with a less than 3-fold difference in the percentage radioactivity that was cell bound ([26]; D. M. Goldenberg and M. J. Mattes, unpublished work). Therefore the large difference observed with LL1 can be attributed to the continuous very rapid catabolism of this Ab.

The supernatants in the LL1 experiments were analysed for catabolic products, by precipitation with methanol. It would be expected that with a label such as conventional ^{125}I , which is rapidly released from the cell after Ab degradation, catabolic products would be present in the supernatant. This in fact was observed, as shown in Figure 4. There was also significant amounts of catabolized indium in the supernatant: 14.6% at 24 h and 28.4% at 48 h. Thus the indium-labelled catabolic products are not totally retained within the cells, but rather are excreted slowly, which is consistent with previous data [14]. Control experiments, described in the Materials and methods section, demonstrated that the radiolabelled Abs were stable in tissue culture medium at 37°C , so the catabolism must be due to cell metabolism. If the amount of Ab 'handled' by the cells is calculated, by adding the percentage cell bound to that catabolized and released, this should be the same for both radiolabels, if the immunoreactivity is the same. In fact, this was observed for the ^{125}I and ^{111}In labels (Figure 4). Thus the rapid Ab catabolism is clearly seen with a conventional iodine label, but only if the supernatant is analysed.

The percentage of bindable radioactivity was determined using a large number of target cells to achieve antigen excess. These experiments were performed with the indium label and a 1 h incubation at 37°C , which is short enough for significant release of catabolic products not to occur. Some 60.6% of the indium radioactivity was specifically bound, and a plateau was reached in the percentage bound, as the cell number was increased, indicating that the percentage bindable determined was reliable. Since approx. 59% of the radioactivity in the wells was handled by the cells in the experiment described in Figure 4, we can conclude that virtually all of the active Ab present in the wells was bound to the cells, internalized and catabolized over a 2-day period.

To determine the capacity of this mechanism for Ab transport, similar experiments were set up with increasing Ab concentrations, up to $10 \mu\text{g/ml}$. In this way, we hoped to saturate the Ii expressed on the cell surface. Although it is difficult to bind every Ii molecule appearing, considering that they reside on the surface for a short time, this approach can provide at least a minimum estimate of the number of Ii molecules processed. Cell counts were obtained at each time point, so that we could calculate Ab molecules handled per cell. These experiments were performed with an indium label. The total Ab molecules handled was again calculated from the cell-bound radioactivity plus the degraded radioactivity in the supernatant; with the indium label, the great majority of the handled radioactivity is cell bound at

Figure 5 Processing of ^{111}In -LL1 by Raji cells, in a prolonged Ab incubation

(A) and (B) Cells were incubated with various concentrations of Ab for the time indicated. Circles, 10 $\mu\text{g/ml}$; squares, 5 $\mu\text{g/ml}$; triangles, 2.5 $\mu\text{g/ml}$; inverted triangles, 1.25 $\mu\text{g/ml}$. (A) The percentage of the total radioactivity in the wells that was cell-bound (filled symbols) or degraded and released into the supernatant (open symbols). These percentages decreased as the Ab concentration was increased, showing the limited (but still large) capacity of the uptake mechanism. In control experiments with a non-reactive ^{111}In -Ab, only 0.1% was cell-bound at 24 h and $0.4 \pm 0.1\%$ at 48 h, so the great majority ($> 95\%$) of cell-bound Ab was antigen-specific, even at the highest Ab concentration. (B) The number of Ab molecules 'handled' per well is shown, for each Ab concentration. 'Handled' Ab is the sum of the cell bound Ab plus the Ab that has been degraded and released by the cells. The amount of Ab handled increases with increasing Ab concentration, and increases at a linear rate over the 2 days of the experiment. In control experiments, a non-reactive ^{111}In -Ab was handled at a rate of approx. 0.5% per day, so at least 90% of the Ab handled was antigen-specific, even at the highest Ab concentration. (C) Ab molecules handled per cell over 26 h as a function of Ab concentration. Cell counts were obtained at each time point. A plateau is being approached, but not yet reached. Values shown are means of duplicates, and standard deviations were all less than 2% of the total radioactivity/well. Correction for non-specific Ab handling had a small effect, with 6.3×10^6 – 7.4×10^6 Ab molecules specifically handled per cell in 26 h, with the Ab at 10 $\mu\text{g/ml}$. This correction would affect results with high Ab concentrations more than with lower Ab concentrations (since the percentage radioactivity bound decreases as the Ab concentration increases), so would cause a flattening of the plateau. Results shown are representative of four experiments.

24 h. We also determined the uptake and processing of a control non-reactive Ab, labelled identically; with this Ab, the uptake was very low, as described in the Materials and methods section.

Figure 5 shows the results of these experiments. Figure 5(A) shows the radioactivity obtained, and Figure 5(B) shows the calculated Ab molecules handled per well. The percentage uptake of course decreases as the Ab concentration increases. Figure 5(B) demonstrates that the number of Ab molecules handled increases with increasing Ab concentrations, and that Ab uptake proceeds at a constant rate, dependent on the Ab concentration. Figure 5(C) shows the number of Ab molecules handled/cell per day (actually 26 h) as a function of Ab concentration. A plateau was approached as the Ab concentration increased up to

10 $\mu\text{g/ml}$, suggesting that most of the Ii was bound by Ab. Approx. 8×10^6 molecules of LL1 were processed/cell per day. Since the cells were dividing during the culture period, and since the cell counts were obtained at the end of each incubation period, the actual number of Ab molecules processed per cell is somewhat higher.

DISCUSSION

We have demonstrated that B-cell lymphomas take up and catabolize nearly 10^7 molecules of LL1 per cell per day. Although LL1 is a new Ab to Ii, our results are entirely consistent with those obtained with Ab BU45 [12], so are not dependent on the particular Ab. This catabolic rate is much faster than that observed with other Abs that are generally considered to be 'rapidly internalized', such as Abs to CD19, CD22 and the transferrin receptor, as shown in Figure 2. We note that our results with these other Abs are very similar to those obtained by other laboratories [27]. In the case of the transferrin receptor, it is known that the Ab is rapidly internalized, within an hour, but generally recycles to the cell surface without being degraded [28]. Other Abs that show rapid catabolism are Abs to growth factor receptors, such as the epidermal growth factor (EGF) receptor [29]. However, these Abs are very different from LL1 in the extent of Ab uptake. With anti-(EGF receptor), as with anti-(transferrin receptor) and anti-CD22 [28,30,31], receptor expression is down-regulated after Ab binding, so large amounts of Ab will not be transported into the cell. Also, unlike LL1, rapid uptake of anti-(EGF receptor) Abs requires the cross-linking generated by bivalent Ab binding. LL1 uptake does not require cross-linking, since monovalent Fab' fragments were processed similarly to the whole Ab.

Similarly rapid uptake of surface-bound proteins occurs for ligands binding to receptors that are internalized via coated pits [32–35]. It seems very likely that LL1 uptake is via coated pits, although this remains to be demonstrated directly. Goldstein et al. [32] described four pathways which endocytosed material could take after internalization via coated pits; the class II–Ii pathway is similar in some respects to the other pathways, yet appears to have two unique properties. First, the protein expressed on the cell surface does not bind to a ligand, so this can hardly be classified as 'receptor-mediated endocytosis', a term often used synonymously with 'clathrin-dependent endocytosis'. Secondly, the proteolytic compartment (the lysosome or pre-lysosome) is not the end stage, but rather the site where the mature class-II molecule is generated. Presumably peptide binding induces a conformational change that is recognized by the transport mechanism responsible for delivery of mature class-II molecules back to the cell surface, as has been suggested [5]. The example of clathrin-dependent endocytosis which seems most similar to Ii uptake is IgA transport across epithelial cells from interstitial fluid to secretions, in that these receptors, the polymeric Ig receptors, also are used only once, and are cleaved as part of the transport mechanism [36]. In contrast with the polymeric Ig receptors and EGF receptor, receptors such as the transferrin receptor, the low-density lipoprotein receptor, and the asialoglycoprotein receptor release the ligand in a low-pH compartment after internalization, and then recycle to the cell surface, so the same receptor molecule can transport many molecules of ligand [32–35]. The rate of ligand uptake in these cases is similar to the rate described here for LL1. However, since Ii probably delivers only a single Ab molecule to lysosomes, unlike the recycling receptors, the rate of synthesis of Ii must be much greater than that of the other receptors mentioned.

In interpreting our results, a key question is whether the

processing of Ab LL1 is a reflection of the processing of the Ii antigen. This would not be the case if the antigen and Ab dissociate intracellularly, and thereafter follow separate pathways. However, this must be considered unlikely, primarily because typical antigen–Ab complexes will not dissociate at the level of acidity encountered intracellularly, pH 4–5 [37]. In our experiments, no significant LL1 Ab was eluted from the surface of Raji cells by pH 4.0 acetate buffer. We note that some evidence suggests that a proportion of cell surface Ii molecules may recycle back to the cell surface after internalization [2]. However, such recycling would not affect either the results or the conclusions of these experiments, if the Ab remains bound to the Ii during recycling, as has been demonstrated with Abs to the transferrin receptor [28] and the FcRII receptor [37]. Results of Roche et al. [12] support the idea that internalized Ii rapidly enters a catabolic compartment. First, they found that Ii labelled with ^{125}I on the cell surface by the lactoperoxidase method was originally complexed with the class-II $\alpha\beta$ -chains, but became largely separated within 2 h. Second, their experiments with brefeldin suggest that Ii, unlike the transferrin receptor, was catabolized after internalization, since brefeldin is considered to block the delivery of newly synthesized proteins to the cell surface, but not to block recycling [38]. Koch et al. [10] had previously also demonstrated that cell surface Ii is associated with $\alpha\beta$ -chains. For these reasons, it seems reasonable to assume that each molecule of LL1 is transported by a molecule of $\alpha\beta$ -Ii to a proteolytic compartment where both the Ab and Ii, but not the class-II $\alpha\beta$ -chains, are catabolized.

Although we have not attempted to follow the biosynthetic pathway of class-II molecules, the data presented have implications with regard to this pathway, specifically with regard to whether all immature molecules reach the cell surface. The synthetic rate, the catabolic rate and the steady-state number of mature molecules per cell must be consistent with each other if most immature forms are efficiently converted into mature forms, which appears to be the case [1,4,6]. Our data suggest that at least 8×10^6 class-II molecules/cell per day are produced via the cell surface route. This is likely to be an underestimate, since not all Ii molecules at the cell surface will have bound Ab before they were internalized, because of their short residence time at the cell surface. Roche et al. [12] calculated a similar rate of transit to the cell surface, of approximately 4.3×10^6 molecules/cell per day with an Epstein–Barr-virus-transformed B-cell line, based only on the half-life of internalization and the number of cell surface molecules per cell. If the half-life of mature class-II molecules present on the cell surface is taken to be approx. 2 days, as determined by Davis and Cresswell [17] with an Epstein–Barr-virus-transformed B-cell line, we can predict a steady-state level of mature class-II molecules per cell of 1.6×10^7 , at which level the catabolic rate would equal the synthetic rate. The measured number of mature class-II molecules expressed per cell is in fact considerably less than this. For Raji cells, the cell line used in most of our experiments, 1.1×10^6 HLA-DR Ab-binding sites per cell were reported [22]. Roche et al. [12] similarly reported approx. 10^6 HLA-DR molecules per cell on the surface of the B-cell line .45, which, as noted above, appears to be quite similar to Raji in its rate of uptake of anti-Ii Ab. Based on these calculations, it appears that the immature molecules directed to the cell surface are sufficient (or more than sufficient) to account for the cell content of mature molecules. Admittedly, however, it is difficult to prove directly that all immature class-II molecules appear on the cell surface. We speculate that transport to the cell surface allows immature class-II molecules to enter early endosomes, and thus to be mixed with unprocessed endocytosed soluble antigens. Therefore antigenic peptides, which will be

unstable, are generated at the same time that Ii is cleaved and the peptide-binding site exposed, which occurs when endocytosed vesicles reach an acidic proteolytic compartment.

An important remaining issue is whether other cell types expressing class-II molecules similarly express Ii at the cell surface which is rapidly internalized. Expression of Ii on Langerhans cells [9] and weak expression on monocytes [11] have been described, but it has also been suggested that some cells with intracellular Ii do not express it on their surface [10]. LL1 was previously reported to react strongly with normal human blood monocytes, and to react very similarly to anti-(class-II $\alpha\beta$ -chain) Abs in immunohistology of normal human tissues [18]. This issue should be re-investigated, using a residualizing radiolabel on cells of macrophage lineage.

The effect of low temperature on these experiments should be noted, primarily because low-temperature Ab incubations are routinely used in studies of this type. Although low-temperature Ab incubations have certain attractive features, our data and previous data from others strongly suggest that it cannot be assumed that low temperature has no effect other than to reversibly ‘immobilize’ the cell. Kaplan and Keogh [25] demonstrated that a brief low-temperature incubation caused macrophages to shed a large fraction of their membrane in the form of small vesicles. Lin et al. [39] and Loor and Hagg [40] demonstrated that low temperature caused the microvilli on B-lymphocytes to disappear. Our data demonstrate that Ab-processing experiments can be markedly affected by a brief low-temperature Ab incubation. We therefore suggest that the optimal temperature for experiments of this type is 37 °C. Although a disadvantage of this temperature is that processing will occur continuously during the Ab incubation and washes, this effect can be taken into consideration and this disadvantage is outweighed by the assurance that artifacts do not occur.

Ab LL1 may have several useful applications. Since this Ab allows rapid delivery of large numbers of molecules to lysosomes, it may be used to deliver toxins, drugs or radioisotopes that can kill tumour cells expressing cell surface Ii, such as B-cell lymphomas and other cell types. In addition, such toxic conjugates may be useful for immunosuppression *in vivo*, since they can potentially kill all cells expressing Ii on their surface, which may include all antigen-presenting cells. Since internalized LL1 is probably present at precisely the time at which peptide-binding sites on class-II molecules are loaded, it may also be useful for the efficient delivery of particular antigenic peptides, such as LL1–antigen or LL1–peptide conjugates, to specifically enhance the immune response. The Ab may also be useful for analytical purposes, to determine whether a particular radiolabel, for example, is trapped in lysosomes, since delivery to lysosomes is so rapid and complete. In the past, the most frequently used system for such analysis has been the delivery of asialoglycoproteins to liver cells, but it would clearly be an advantage in many cases to use B-cell lymphomas for this purpose.

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