Immunology 1995 **84** 164–170

Modulation of antigen processing and presentation by covalently linked complement C3b fragment

M. R. JACQUIER-SARLIN,* F. M. GABERT, M.-B. VILLIERS & M. G. COLOMB *CEA, ICH, Unité INSERM 238, DBMS, Centre d'Etudes Nucléaires de Grenoble, Grenoble, France*

SUMMARY

Ligands such as complement fragments (C3, C4), IgG or α_2-macroglobulin, which bind antigen (Ag) before their uptake by antigen-presenting cells (APC), are likely to modulate the different steps of Ag processing and presentation. These ligands contribute to internalization and endosomal targeting of Ag; they also influence its processing and, consequently, the binding of resulting peptides to major histocompatibility complex (MHC) class II molecules before presentation to T cells. Complement protein C3 contains, like other members of the α_2-macroglobulin family, an intrachain thiolester bond. Conformational alteration or limited proteolysis of C3 into C3b leads to breaking of the thiolester with transient capacity of the revealed carbonyl group to esterify hydroxyl groups of Ag. Ester-linked complexes including tetanus toxin (TT) and C3b were prepared to analyse the influence of bound C3b on TT processing and presentation by APC. Covalent binding of C3b to TT resulted in increased and prolonged stimulation of specific T-cell proliferation. This effect was observed with non-specific B cells, as well as with a TT-specific B-cell clone, as APC. On the other hand, SDS–PAGE analysis of proteolysates of TT or C3b–TT, obtained with endosome/lysosome-enriched subcellular fractions prepared from human Epstein–Barr virus (EBV)-transformed B cells, indicated a delay of TT proteolysis when TT was associated to C3b. Treatment of APC with protease inhibitors, before and during exposure of the cells to Ag, resulted in differences in the inhibition of TT and C3b–TT proteolysis. Using purified cathepsins B and D, we demonstrated that covalent binding of C3b to TT totally abolished TT proteolysis by cathepsin D, while proteolysis by cathepsin B was preserved. This finding and the absence of cathepsin B in endosomes may explain a delay in TT processing when it is associated to C3b. Confirming these data, presentation by formaldehyde-fixed cells of C3b–TT proteolysates showed higher stimulation of specific T-cell clones than formaldehyde-fixed TT proteolysates.

INTRODUCTION

The antigen (Ag)-specific immune response is largely controlled by T lymphocytes, which recognize Ag only after they have been processed by antigen-presenting cells (APC; B lymphocytes, macrophages, dendritic cells, etc.) and complexed with major histocompatibility complex (MHC) molecules. Processing of exogenous Ag occurs after their internalization, followed by several intracellular steps generating peptides that can then combine with MHC class II molecules. These steps usually involve Ag denaturation,[1,2] reduction[3,4] and/or limited proteolysis.[5] Peptide–MHC class II complexes migrate to the cell surface, where they contribute to specific T-cell triggering.[6] Processing depends on several factors, such as the concentration and the structure of extracellular Ag,[7] its capture by APC, the intracellular proteolytic potential and the stability of peptide–class II complexes. Class II expression and Ag uptake have been studied in many different APC: low Ag concentration can be compensated for by high class II molecule expression,[8] or by increased Ag uptake by APC (targeting of Ag to APC surface receptors such as membrane immunoglobulin and complement receptors).[9–11] This is supported by the recent evidence that there is involvement of α_2-macroglobulin receptors in enhanced Ag presentation.[12]

The influence of proteinic assistance on the intracellular traffic and processing of exogenous Ag in APC, has been less well documented but has been described for heat-shock protein 70 (hsp 70).[13] Greater consideration should be given to ligands

Received 13 July 1994; revised 13 September 1994; accepted 16 September 1994.

Abbreviations : APC, antigen-presenting-cells; EBV, Epstein–Barr virus; TT, tetanus toxin.

*Present address: Unité d'Allergologie, Hôpital Cantonal Universitaire de Genève, 24, rue Micheli-du-crest, CH-1211 Geneve 4, Switzerland.

Correspondence: Dr M. G. Colomb, CEA, ICH, Unité INSERM 238, DBMS, Centre d'Etudes Nucléaires de Grenoble, 17 rue des Martyrs, F38054, Grenoble Cedex 9, France.

such as IgG, α_2-macroglobulin and C3 and C4 fragments, which bind Ag before their uptake by APC. Besides their established role in internalization and endosomal targeting of Ag, these ligands are likely to influence the processing of Ag and, consequently, the binding of resulting peptides to MHC class II molecules before presentation to T cells.

Complement protein C3 is a member of the α_2-macroglobulin family, with an intrachain thiolester bond.[14] Breaking of this bond, by conformational change or limited proteolysis, results in a capacity for covalent binding of C3b to diverse proteins.[15,16] We have previously demonstrated that C3b is able to bind soluble Ag such as tetanus toxin (TT).[17,18] Two types of Ag–C3b complexes were obtained: (1) ester (or accessory amide) complexes, which involve the carbonyl moiety of the thiolester; and (2) disulphide complexes, which involve the SH group of the thiolester.

We report here the first evidence for a role of C3b covalently linked to TT in enhancing TT peptide presentation to specific T-helper (Th) cells. TT-associated C3b delays toxin proteolysis, contributing to a more gradual generation of immunogenic peptides. The modulatory role of C3b in Ag processing was analysed on TT and C3b–TT complex proteolysis and on the TT peptide-induced T-cell proliferation.

The role of Ag-bound C3b has for a long time been attributed totally to non-covalent interactions of C3 fragments with cellular complement receptors (CR1, CR2, CR3, depending on the cell) and subsequent enhanced capture of C3b-opsonized Ag. We now propose that covalent binding of C3b to TT also modulates intracellular processing of TT and plays a significant role in Ag presentation.

MATERIALS AND METHODS

Reagents and cell-culture media

Complete RPMI (RPMIc) was RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate (Gibco, Cergy-Pontoise, France), 50 mM 2-mercaptoethanol, 1% non-essential amino acids (Gibco) and kanamycin (100 μg/ml; Gibco). RPMIc/FCS was RPMIc supplemented with 10% heat-inactivated fetal calf serum. RPMIc/HS was RPMIc supplemented with 10% heat-inactivated human serum.

Antigens and cells

Native TT was generously donated by Dr J. R. Cartier (Institut Mérieux, France) and was further purified as described previously.[17] C3b–TT complexes were prepared according to Villiers *et al.*[17] Purified TT was labelled with ^{125}I using the iodogen method.[19] Free iodine was eliminated by filtration through a Sephadex G50-fine column. Epstein–Barr virus (EBV)-transformed human B cells were used as APC. The Ag non-specific B-cell line (LCM) was derived from a donor, CM (DR3/DR11); the TT-specific B-cell clone, 4.2 (DR3/DR11), was kindly provided by Dr A. Lanzavecchia.[20] The T-cell line TCM2 and its derived T-cell clones specific for TT were obtained as described elsewhere.[21] Briefly, peripheral blood mononuclear cells from a TT-immune donor (DR3/DR11) were stimulated in 2 ml of RPMIc/HS by 20 μg/ml of TT in 24-well plates (Falcon, Becton Dickinson, Le pont de Claix, France). After 7 days, the T cells were placed on a discontinuous Percoll gradient (Pharmacia, St Quentin en Yvelines, France) and separated according to density. The blast cells were collected in a single band and cultured in RPMIc/HS supplemented with 25 U/ml recombinant interleukin-2 (IL-2; Vie 3000), with changes in medium every 3–5 days. Following 3–4 weeks in culture, T cells were restimulated with TT and autologous irradiated (3500 rads) peripheral blood mononuclear cells. After a further expansion of TCM2 T cells in IL-2, the cells were cloned by limiting dilution (0·2 cell/well) in the presence of irradiated heterologous peripheral blood mononuclear cells (3500 rads), phytohaemagglutinin A (PHA; 2 μg/ml; Sigma, La Verpillière, France) and IL-2 (50 U/ml). A TCM2 T-cell clone specific for TT, TCM2-10, was cultivated by periodic stimulations with PHA, in the presence of irradiated heterologous peripheral blood mononuclear cells, followed by expansion in the presence of IL-2.

Subcellular fractionation and cathepsin identification

Endosome/lysosome-enriched fractions were obtained by differential centrifugation of 4.2 cell lysate in homogenization buffer (pH 7·4) containing 0·25 M sucrose, 1 mM EGTA and 3 mM Imidazole, as described elsewhere.[22] Further separation of these two subcellular compartments in Percoll gradient was according to a modified protocol of Stoorvogel *et al.*[23]

Cathepsin B and D activities were determined using benzyloxycarbonyl-Arg-Arg-2-naphthylamide and benzoyl-Arg-Gly-Phe-Phe-Pro-4-methoxy-2-naphthylamide (20 mM in 50 mM acetate buffer, pH 5; Bachem, Interchim, Montfuçon, France), respectively, as substrates. Twenty microlitres of each fraction from the Percoll gradient was mixed with 75 μl 50 mM acetate buffer (pH 5) containing 0·01% (w/v) Triton X-100 and 6 mM cysteine. After addition of substrate (5 μl) and 1 hr incubation at 37°, the reaction was stopped with 1 mM iodoacetamide (950 μl). Release of 2-naphthylamide was monitored by its fluorescence at 410 nm (excitation = 335 nm) using a F-2000 Fluorescence Spectrophotometer (Hitachi, Les Ulis, France).

T-cell proliferation assays

2×10^4 LCM or 4.2 B cells irradiated at 10 000 rads were incubated in 96-well flat-bottomed microtitre plates (Falcon) with Ag—TT (66 nM) or C3b–TT (0·66 nM) and 2×10^4 TCM2-10 cells in a final volume of 200 μl. After 48 hr, the cultures were pulsed for 20 hr with 1 μCi of [^{3}H]thymidine and the incorporated radioactivity counted. Cultures were in triplicate, including a blank without Ag. For experiments with protease inhibitors, 2×10^4 B cells (4.2) were preincubated in 96-well flat-bottomed microtitre plates (Costar) in the presence or absence of different protease inhibitors for 6 hr before addition of TT (66 nM) or C3b–TT (0·66 nM).

In TT proteolysate presentation to the TCM2-10 cell clone, 4.2 cells were fixed according to Casten & Pierce.[24] 2×10^4 fixed cells were incubated in 96-well round-bottomed microtitre plates (Costar) in the presence of 50 μl digested TT or C3b–TT for 2 hr at 37°. The plates were centrifuged before addition of 2×10^4 TCM2-10 T cells. Proliferation of these cells was analysed as described before.

In vitro proteolysis of ^{125}I-TT or ^{125}I-TT–C3b by purified cathepsins and endosome/lysosome-enriched B-cell subcellular fractions

^{125}I-TT or ^{125}I-TT–C3b (0·1 μM) was incubated at 37° for different times in the presence of 5 μg cathepsin B (Novabiochem,

France Biochem, Meudon, France) or 10 μg cathepsin D (Novabiochem), in 50 mM sodium acetate buffer, pH 5·5 (final volume 50 μl). For cathepsin B, sodium acetate buffer was 8 mM in L-cysteine to optimize thiol proteolysis. The reactions were stopped by adding 10 mM iodoacetamide (cathepsin B) or 10 mM pepstatin A (cathepsin D), followed by a further incubation of 30 min at 37° before cooling to 0°. Proteolysates were analysed by SDS–PAGE (8% acrylamide gels) under non-reducing conditions;[25] the gels were then dried and exposed to Hyperfilm-MP (Amersham, Amersham, UK) at −70°. Ag proteolysis by B-cell endosome/lysosome-enriched subcellular fractions was analysed as follows: 1 μg ^{125}I-TT or ^{125}I-TT–C3b was incubated for different times at 37° in the presence of 30 μl of endosome/lysosome-enriched fractions prepared from 10×10^6 4.2 B cells in 50 mM sodium acetate buffer, pH 5·5 (final volume 50 μl). The proteolysis was stopped by cooling samples to 0°, before analysis of the proteolysates as described before.

Proteolysis of ^{125}I-TT by TT-specific B-cell clone 4.2

10×10^6 4.2 cells were incubated at 4° for 1 hr in the presence of ^{125}I-TT (0·1 μM). After centrifugation to eliminate unbound TT, cells were then incubated in RPMI containing 20 mM HEPES for different times at 37°. The proteolysis was stopped by cooling samples to 0°. Membrane-associated ^{125}I-TT was removed by acidic treatment (25 mM glycine, 150 mM NaCl, pH 2·75) during 15 min at 4°; the cells were then lysed in phosphate-buffered saline containing 0·1% Nonidet P-40, 200 U/ml aprotinin and 5 mM di-isopropyl fluorophosphate (DFP). Lysates were analysed by SDS–PAGE (8% acrylamide gels) under non-reducing conditions as described before.

RESULTS

C3b binding to TT up-regulates the B-cell mediated presentation of TT peptides to T cells

The influence of covalent binding of C3b to TT on Ag presentation was assessed first. The ability of TT alone or preformed TT–C3b complexes (C3b–TT) to induce human TT-specific TCM2 T-cell line proliferation was compared. Presentation of TT was analysed using two types of EBV-transformed B cells [the Ag non-specific line (LCM) or the TT-specific clone (4.2)] (Fig. 1). Both TT and C3b–TT induced a dose-dependent TCM2 T-cell response when they were presented by LCM or 4.2 cells. TT presentation by the specific B-cell clone 4.2 was more efficient than TT presentation by the non-specific B-cell line LCM. As expected, the efficiency of C3b–TT over TT to stimulate T cells was higher with presentation by the non-specific LCM cells: a similar T-cell stimulation was observed with 100-fold less C3b–TT than TT with the LCM line as APC, or with 10-fold less C3b–TT than TT with 4.2 cells as APC.

Covalent binding of C3b to TT slows down the proteolysis of TT by subcellular fractions from presenting cells

The favoured TT presentation by associated C3b observed even with the specific 4.2 B cells suggested that associated C3b was likely to modify Ag proteolysis in the APC. As a first line of investigation, we compared the proteolysis of TT and C3b–TT by APC subcellular fractions.

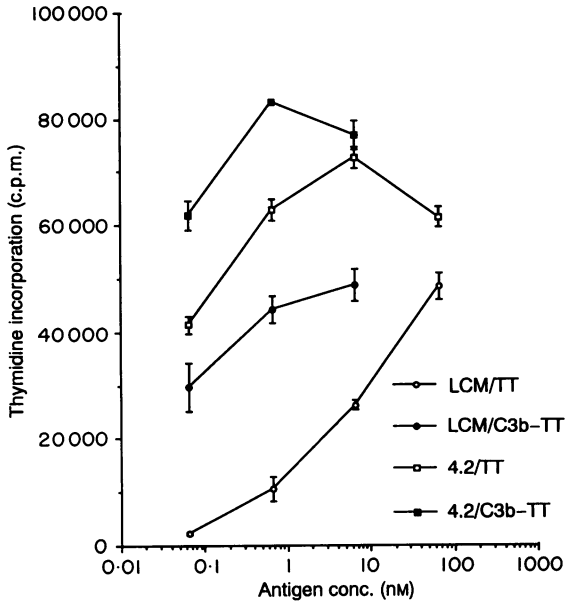

Figure 1. Comparative efficiency of the non-specific B-cell line (LCM) or the TT-specific B-cell clone 4.2 to present TT or C3b–TT. 2×10^4 TCM2 cells were cultured with 2×10^4 irradiated (10 000 rads) EBV-transformed B cells, in the presence of increasing concentrations of TT or C3b–TT. T-cell proliferation was estimated, as described in the Materials and Methods.

Proteolysis of Ag by endosome/lysosome-enriched subcellular fractions prepared from the human B-cell clone 4.2 was analysed by SDS–PAGE. Kinetic analysis of fragments released from ^{125}I-TT and C3b–^{125}I-TT complexes (Fig. 2a, b) revealed an inhibition of TT proteolysis of more than 50% when TT was associated with C3b; this inhibition was evaluated from the amount of uncleaved TT, as shown in Fig. 2b. There was no apparent alteration in the specificity of TT cleavage from the proteolytic patterns of TT and C3b–TT, as proteolytic fragments of similar sizes were detected on SDS–PAGE (Fig. 2a). The major products were 135 000 and 120 000 MW fragments, with smaller fragments migrating at the front.

Cathepsin B- and D-mediated TT proteolysis is inhibited by associated C3b

Inhibition of TT proteolysis by bound C3b may result from reduced accessibility or availability of TT to various intracellular proteases. To test this hypothesis, the ability of non-cytotoxic protease inhibitors to block the toxin presentation to the TCM2-10 clone was analysed (Fig. 3). Puri & Factorovich[26] have previously demonstrated that these inhibitors are effective against Ag processing at low concentration, as their small mw facilitates their penetration into presenting cells. Among the five inhibitors tested, three inhibited thiol proteases (leupeptin 235 μg/ml, E-64 100 μg/ml, antipain 200 μg/ml), one inhibited aspartyl proteases (pepstatin A 100 μg/ml) and one serine proteases (chymostatin 100 μg/ml). To optimize their action, each inhibitor was first preincubated for 6 hr with APC, before

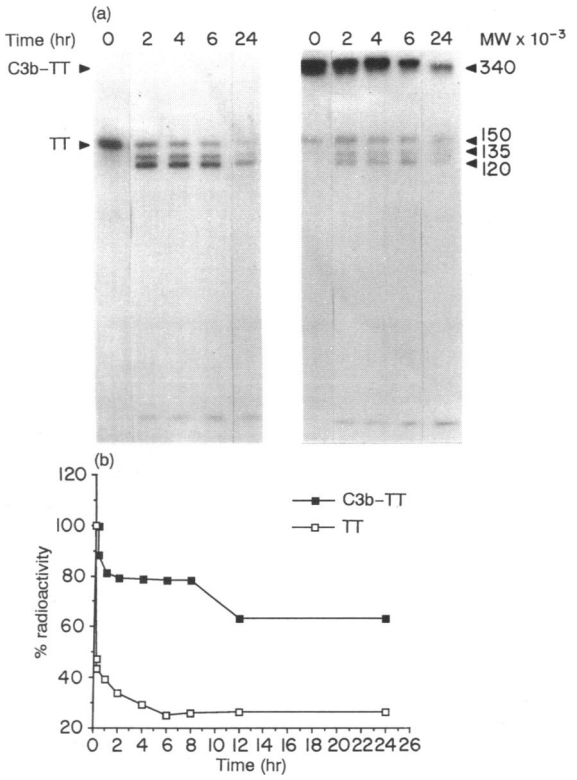

Figure 2. Kinetic analysis of TT or C3b–TT proteolysis by endosome/lysosome-enriched subcellular fractions from the B-cell clone 4.2. (a) SDS–PAGE analysis of TT (left) or C3b–TT (right) proteolysates obtained after 0, 2, 4, 6 and 24 hr of incubation at 37°, as described in the Materials and Methods. (b) Percentage uncleaved TT calculated from gel radioactivity scanning, as a function of time.

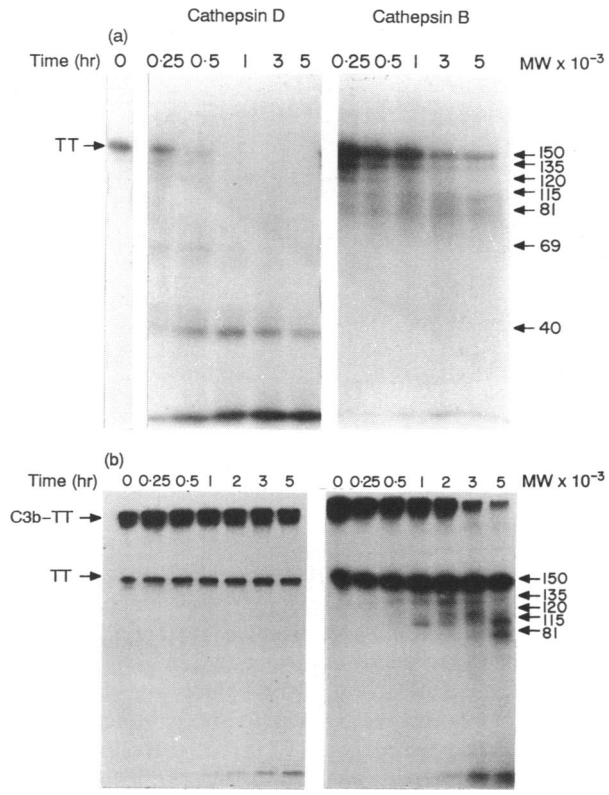

Figure 4. Kinetics of TT or C3b–TT proteolysis by purified cathepsins. (a) SDS–PAGE analysis in non-reducing conditions of TT digests by cathepsins D and B, at 15 min, 30 min, 1, 2, 3 and 5 hr at 37°. (b) SDS–PAGE analysis in non-reducing conditions of C3b–TT digests by cathepsins D and B, at 15 min, 30 min, 1, 2, 3 and 5 hr at 37°.

Ag addition (TT at 66 nM and C3b–TT at 0·66 nM). In each case, the results are expressed as the percentage of T-cell response in the absence of inhibitors.

As illustrated, inhibitors like leupeptin inhibited proteolysis

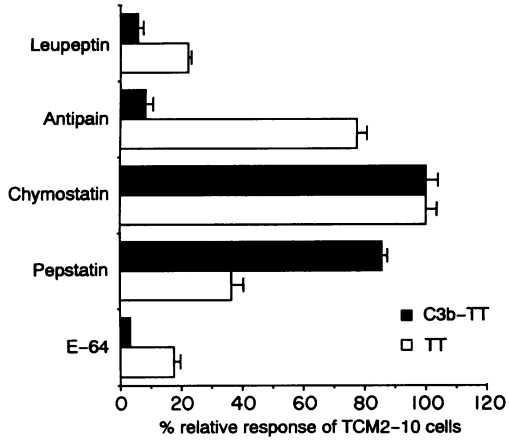

Figure 3. Effect of protease inhibitors on TT or C3b–TT presentation by B-cell clone 4.2 to TCM2-10 cells. 2×10^4 irradiated (10 000 rads) EBV-transformed B cells (clone 4.2) specific for TT were preincubated in the presence of different protease inhibitors for 6 hr. Then TT (66 nM) or C3b–TT (0·66 nM) was added. After 48 hr of culture with 2×10^4 TCM2-10 cells, T-cell proliferation was estimated from [^{3}H]thymidine incorporation, as described in the Materials and Methods.

of TT as well as C3b–TT by 78% and 94%, respectively, while others were more specific: antipain inhibited only the presentation of C3b–TT (92% inhibition); pepstatin A blocked TT presentation by 64% and C3b–TT presentation by 12%. Chymostatin did not alter presentation of either Ag. The addition of each inhibitor after TT or C3b–TT processing did not modify Ag presentation to T cells, suggesting that: (1) thiol and aspartyl protease inhibitors act at the level of processing, most probably on proteases such as cathepsin B and D; and (2) these inhibitors are not toxic for T-cell proliferation.

For further characterization of proteases involved in Ag processing, we carried out kinetic studies of TT or C3b–TT processing by purified cathepsins D and B, and confirmed the purity and the specificity of these two proteases by addition of leupeptin (for cathepsin B) or pepstatin A (for cathepsin D) in control tests (data not shown). As shown in Fig. 4a, these two cathepsins were able to digest TT alone but with different kinetics: the proteolysis by cathepsin D was more rapid than the proteolysis by cathepsin B. The major proteolytic products were also different: 69 000 and 40 000 MW fragments and peptides which migrated at the front upon SDS–PAGE for cathepsin D, major fragments of 135 000, 120 000, 115 000 and 81 000 MW for cathepsin B. The covalent association of C3b to TT completely protected TT from proteolysis by cathepsin D, as shown in Fig. 4b. In contrast, cathepsin B was still active with C3b–TT, with the generation of similar fragments.

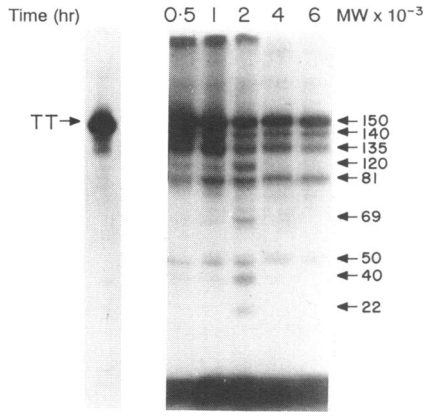

Figure 5. Kinetics of TT intracellular proteolysis in 4.2 B cells. SDS–PAGE analysis in non-reducing conditions of TT digests after endocytosis by 4.2 cells for 30 min, 1, 2, 4 and 6 hr at 37°.

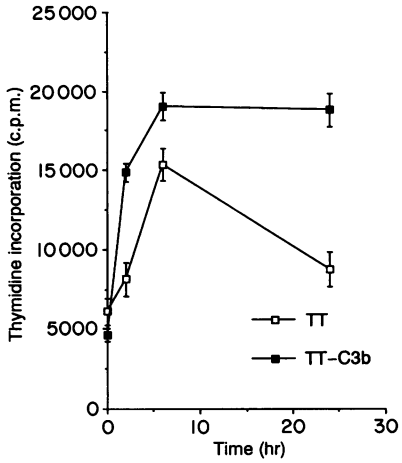

Figure 7. Presentation of TT or C3b–TT digests to T cells by fixed B cells. Conditions were as described in the Materials and Methods.

However, the 135000 and 120000 MW bands that were quite dense at 15 min with TT alone, were not even apparent until 30 min with C3b–TT and were prominent at 2 hr. Cathepsin B processing of TT covalently associated to C3b seems to involve two steps: (1) esterolysis of C3b–TT complexes, releasing free toxin; (2) proteolysis of the available free toxin. Kinetic analysis of the intracellular proteolysis of TT in 4.2 live B cells, indicated that *in vitro* proteolysis of ^{125}I-TT by purified cathepsins mimicked the intracellular processing of this Ag (Fig. 5). In this case, Ag degradation occurred between 15 and 30 min after internalization, with the production of major bands at 140000, 135000, 120000, 81000, 69000, 40000 and 20000 MW, in addition to small peptides. These results confirmed involvement of cathepsins B and D in TT and C3b–TT processing.

The intracellular localization of cathepsins B and D was

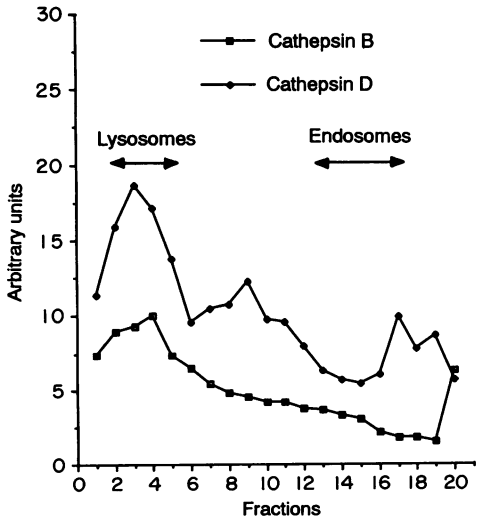

Figure 6. Subcellular localization of cathepsins D and B. Cells were lysed and fractionated on Percoll gradient as described in the Materials and Methods. In each fraction cathepsin D and B activities were estimated as described in the Materials and Methods. Endosomes and lysosomes were identified using transferrin (endosomes) and β-galactosidase activity (lysosomes) as markers, their position on the gradient is indicated by horizontal bars.

investigated after Percoll gradient purification of endosomes and lysosomes. Specific markers (transferrin and galactosaminidase) were used to characterize these compartments, which were located in the gradient from fractions 13–17 and 2–5, respectively (data not shown). As represented in Fig. 6, cathepsin D activity was detected in endosomes as well as in lysosomes where the highest peak was observed, whereas cathepsin B activity was detected only in lysosomes. The fact that only cathepsin B was able to process TT in C3b–TT complexes, and its particular localization in lysosomes, explain a transfer of non-degraded C3b–TT complexes in endosomes and thus a delay in TT processing, which starts only in lysosomes.

Delay in C3b–TT proteolysis leads to enhanced specific T-cell proliferation

The link between delayed processing of TT in C3b–TT complexes and improved T-cell proliferation was investigated as follows: TT and C3b–TT complexes were incubated for different times, in the presence of endosome/lysosome-enriched subcellular fractions from B cells. The resulting proteolysates were then presented to TT-specific T cells by formaldehyde-fixed B cells. T-cell activation and proliferation were maximum at 6 hr with proteolysates of TT and then declined, whereas they were still preserved at 24 hr with proteolysates of C3b–TT (Fig. 7). Again, the binding of C3b to TT prolonged and optimized Ag presentation, as reflected by [^{3}H]thymidine uptake in T cells.

DISCUSSION

Beyond the well-known role of C3b in the non-specific immune response, as illustrated by bacterial opsonization and phagocytosis, we present here evidence that covalent binding of C3b to Ag modulates different steps of a specific immune response.

TT associated to C3b was more efficiently presented to T cells than free TT. The efficiency of C3b–TT presentation was best observed with a non-specific B-cell line, LCM; this effect relied in part on the increase of uptake of Ag mediated by C3b receptors present on LCM cells.[9] The influence of C3b

receptors can also occur on other non-specific cells, such as B cells, monocytes or dendritic cells.[27] Enhanced stimulation, but at a lower level, was observed when C3b–TT complexes were presented by the TT-specific B-cell clone 4.2, expressing membrane immunoglobulin specific for TT ($K_d = 10^{-9}$ M) and complement receptors.[18]

Analysis of Ag proteolysis showed that covalent binding of C3b to TT delays toxin proteolysis by subcellular fractions from presenting cells, without affecting the specificity of TT cleavage. Using protease inhibitors we demonstrated that serine and thiol proteases such as cathepsin B or L, and aspartyl proteases such as cathepsin D, are involved in TT processing. These results agree with the observations of Rodriguez & Diment[28] and Diment *et al.*,[29] who showed that cathepsins D and B, respectively, are the major endosomal and lysosomal proteases involved in intracellular Ag cleavage. The involvement of these proteases in TT processing was assessed from *in vitro* proteolysis of TT or C3b–TT in the presence of purified cathepsins: fragments produced *in vitro* by cathepsins B and D were very similar to fragments detected in specific B cells after endocytosis (the apparent molecular weight of the major fragment was 135 000, 120 000, 81 000, 69 000 and 40 000 MW) (Figs 4 and 5). However, differences were observed between the two cathepsins: (1) in their kinetics, (2) in the size of some fragments produced and, more importantly, (3) in their different ability to proteolyse TT in TT–C3b complexes. In fact, free TT was more rapidly degraded by cathepsin D than cathepsin B, whereas C3b-associated TT was proteolysed only by cathepsin B. These results indicate that Ag processing involves several proteases with a concerted cleavage by the main endolysosomal cathepsins.[30]

Taken together, these results lead us to propose that C3b alters the intracellular processing of associated TT in C3b–TT complexes: the Ag is exposed to different proteases as it progresses through cell subcellular compartments. Free TT could be processed by cathepsin D in endosomes, the first compartment it enters, while C3b–TT must reach lysosomes before being processed. The protective role of C3b relies on good stability of the TT–C3b ester bond at acidic pH.[17,31] In lysosomes, cathepsin B contributes to free TT delivery and, together with cathepsin D also present in this compartment, to TT proteolysis. Gradual Ag availability is essential for optimal production of peptides, which can bind MHC class II molecules and stimulate T cells (Fig. 7). Peptides generated in endosomes may bind to class II that are internalized from and recycled to the cell surface,[32] or to newly synthesized class II.[33] Peptides generated in lysosomes bind exclusively to newly synthesized class II.

Comparable experiments, not reported here, have shown that S–S bonding occurs between TT and C3b on TT-specific presenting B cells, involving a membrane thioredoxin-like protein[18] which has been described elsewhere in S–S bond formation catalysis.[34,35] The role of the S–S bond may be similar to that of the ester link, as significant stability of S–S bonds in endosomal fractions was observed.[4] This role is presently under study on TT processing and presentation. A more general role of S–S bonding of C3b to SH-bearing Ag in the handling and presentation of these Ag to T cells can be envisaged.

The abundance of C3 in serum and of C3b in inflammatory areas where various proteinases cleave C3[36] and lead to complex formation with Ag, emphasizes a new biological role for C3b which may be acting as a chaperone. Non-covalent binding of Ag–C3b complexes to complement receptor C3b enhances Ag uptake by APC on one hand, and covalent binding of Ag to C3b optimizes Ag processing and presentation on the other hand. We think that extracellular and intracellular routing of Ag by covalently associated C3b, which could influence Ag processing and presentation, may be of interest in various inflammatory pathologies as well as in engineering new vaccines. This role of C3b could also be shared by the highly polymorphic complement protein C4b, as well as by α_2-macroglobulin.

REFERENCES

1. Allen P.M. & Unanue E.R. (1984) Differential requirements for antigen processing by macrophages for lysosyme-specific T cell hybridomas. *J Immunol* **132,** 1077.
2. Sette A., Adorini L., Colon S.M., Buus S. & Grey H.M. (1989) Capacity of intact proteins to bind to MHC class II molecules. *J Immunol* **143,** 1265.
3. Jensen P.E. (1991) Reduction of disulphide bonds during Ag processing: evidence from a thiol-dependent insulin determinant. *J Exp Med* **174,** 1121.
4. Collins D.S., Unanue E.R. & Harding C.V. (1991) Reduction of disulphide bonds within lysosomes is a key step in Ag processing. *J Immunol* **147,** 4054.
5. Kovac Z. & Schwartz R. (1985) The molecular basis of the requirement for Ag processing of pigeon cytochrome c prior to T cell activation. *J Immunol* **134,** 3233.
6. Unanue E. R. (1984) Ag recognition. *Curr Opin Immunol* **2,** 63.
7. Rouas N., Christophe S., Housseau F., Bellet D., Guillet J.-G. & Bidart J.-M. (1993) Influence of protein-quaternary structure on Ag processing. *J Immunol* **150,** 782.
8. Matis L.A., Longo D.L., Hedrick S., Hannumc., Margoliash E. & Schwartz R.H. (1983) Clonal analysis of the major histocompatibility complex restriction and the fine specificity of Ag recognition in the T cell proliferative response to cytochrome C. *J Immunol* **130,** 1527.
9. Arvieux J., Yssel H. & Colomb M.-G. (1988) Ag-bound C3b and C4b enhance Ag-presenting cell function in activation of human T-cell clones. *Immunology* **65,** 229.
10. Lorenz R.G., Blum J.S. & Allen P.M. (1990) Constitutive competition by self proteins for Ag presentation can be overcome by receptor-enhanced uptake. *J Immunol* **144,** 1600.
11. Lanzavecchia A. (1990) Receptor-mediated Ag uptake and its effect on Ag presentation to class-II restricted T lymphocytes. *Annu Rev Immunol* **8,** 773.
12. Chu C.T. & Pizzo S.V. (1993) Receptor-mediated Ag delivery into macrophages. Complexing Ag to α_2-macroglobulin enhances presentation to T cells. *J Immunol* **150,** 48.
13. Denagel D.C. & Pierce S.K. (1991) Heat shock proteins implicated in Ag processing and presentation. *Semin Immunol* **2,** 65.
14. Dodds A. & Law SK.A. (1988) Structural basis of the binding specificity of the thioester-containing proteins, C4, C3 and alpha-2-macroglobulin. *Complement* **5,** 89.
15. Isaac L. & Isenman D.E. (1992) Structural requirements for thioester bond formation in human complement component C3. *J Biol Chem* **267,** 10062.
16. Barnum S.R. (1989) C3 structure and molecular genetics. In: *Biochemistry and Molecular Biology of Complement* (ed. R. B. Sim), MTP Press, Lancaster, PA.
17. Villiers M.-B., Villiers C.L., Wright J.F., Maison C.M. & Colomb M.G. (1991) Formation of covalent C3b-tetanus toxin

complexes: a tool for the in vitro study of Ag presentation. *Scand J Immunol* **34,** 585.
18. Jacquier M.R., Gabert F.M., Villiers C.L. & Colomb M.G. (1993) Disulfide linkage between C3b and tetanus toxin on tetanus toxin specific EBV-transformed B cells. *J Immunol* **150,** 4253.
19. Salacinsky P.R.P., McLean C., Sykes J.E.C., Clement-Jones V.V. & Lowry P.J. (1981) Iodation of proteins, glycoproteins and peptides using a solid-phase oxidasing agent, 1, 3, 4, 6 tetrachloro-3,6-diphenyl glycoril (iodogen). *Anal Biochem* **117,** 136.
20. Lanzavecchia A., Ferrarini M. & Celada F. (1982) Human T cell lines with Ag specificity and helper activity. *Eur J Immunol* **12,** 468.
21. Lanzavecchia A. (1985) Ag-specific interaction between T and B cells. *Nature* **314,** 537.
22. Darte A.E. & Beaufay H. (1983) Analytical subcellular fractionation of cultivated mouse resident peritoneal macrophages. *J Exp Med* **157,** 1208.
23. Stoorvogel W., Geuze H.J., Griffith J.M. & Strous G.J. (1988) The pathways of endocytosed transferrin and secretory protein are connected in the trans-golgi reticulum. *J Cell Biol* **106,** 1821.
24. Casten L.A. & Pierce S.K. (1988) Receptor-mediated B cell Ag processing. Increased antigenicity of a globular protein covalently coupled to antibodies specific B cell surface structure. *J Immunol* **140,** 404.
25. Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **257,** 680.
26. Puri J. & Factorovich Y. (1988) Selective inhibition of Ag presentation to cloned T cells by protease inhibitors. *J Immunol* **141,** 3313.
27. Levine T.P. & Chain M. (1992) Endocytosis by Ag presenting cells: dendritic cells are as endocytically active as other Ag presenting cells. *Proc Natl Acad Sci USA* **89,** 8342.
28. Rodriguez G.M. & Diment S. (1992) Role of cathepsin D in Ag presentation of ovalbumin. *J Immunol* **149,** 2894.
29. Diment S., Leech M.S. & Stahl P. (1988) Cathepsin D is membrane-associated in macrophages endosomes. *J Biol Chem* **263,** 6901.
30. Guagliardi L.E., Kopperlman B., Blum J.M., Marks M.S., Cresswell P. & Brodsky M. (1990) Co-localization of molecules involved in Ag processing and presentation in an early endocytic compartment. *Nature* **343,** 133.
31. Rey-Millet C.A., Chesne S. & Colomb M.G. (1993) Associated complement C3b towards an understanding of its intracellular modifications. *Mol Immunol* **30,** 855.
32. Harding C.V. & Unanue E.R. (1989) Ag processing and intracellular Ia. Possible roles of endocytosis and protein synthesis in Ia function. *J Immunol* **142,** 12.
33. Neefjes J.F. & Ploegh H.L. (1992) Intracellular transport of MHC class II molecules. *Immunol Today* **11,** 411.
34. Holmgren A. (1985) Thioredoxin. *Annu Rev Biochem* **54,** 237.
35. Pigiet V.P. & Schuster B.J. (1986). Thioredoxin-catalysed refolding of disulphide-containing proteins. *Proc Natl Acad Sci USA* **83,** 7643.
36. Maison C.M., Villiers C.L. & Colomb M.G. (1991) Proteolysis of C3 on U937 cell plasma membranes. Purification of cathepsin G. *J Immunol* **147,** 921.