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Bone marrow pre-B lymphocytes synthesize immunoglobulin μ chains of membrane type with different properties and intracellular pathways

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Mouse normal bone marrow pre-B lymphocytes synthesize only membrane μ chains (μ_m), as shown by mRNA studies and peptide analysis. The μ_m chains exist in two forms: free μ_m chains assembled into dimers, or L chain-bound μ_m chains present in IgM monomers (in the case of 'late pre-B cells', i.e., after productive L chain gene rearrangement). These two forms of molecules are very different in properties, fate and intracellular pathways. Free but not L chain-bound μ chains are highly susceptible to mild proteolysis, which degrades their entire $C\mu_1$ and V_H domains. Free μ chains are rapidly degraded within the lysosomal compartment, which they reach *via* the *cis*, avoiding the *trans*, part of the Golgi complex. In contrast, as soon as μ chains bind to L chains, they are directed towards the '*trans*' Golgi compartment, where they undergo terminal glycosylation, then to the cell surface, where they progressively accumulate. It is suggested that the conformation instability of the $C\mu_1$ and V_H domains of the free μ chains plays a critical role in the intracellular targeting of these molecules, as compared with that of L chain-bound μ chains.

Key words: biosynthetic labelling/intracellular transport/ μ RNA analysis/μ chains/pre-B cells

Introduction

In the adult mouse, B lymphocytes arise from precursors present in the bone marrow, which acquire surface immunoglobulins (sIg^+) when cultured *in vitro* or transferred *in vivo* (Ryser and Vassalli, 1974: Osmond and Nossal, 1974). These precursors, or pre-B lymphocytes, contain cytoplasmic μ heavy chains which are not expressed at the cell surface (Raff *et al.*, 1976; Burrows *et al.*, 1979). The synthesis of μ chains results from the productive rearrangement of their genes, an event which, in B cell ontogeny, precedes the productive rearrangement of L chain genes (Maki *et al.*, 1980; Korsmeyer *et al.*, 1981; Perry *et al.*, 1981; Siden *et al.*, 1981; Alt *et al.*, 1981). Because of the relative difficulty in obtaining pre-B lymphocytes uncontaminated by B lymphocytes, the biosynthesis of Ig chains in pre-B lymphocytes and the secretory (μ_s) or membrane (μ_m) type of their μ chains have been studied to date only in normal fetal liver cells before the emergence of B cells among the hematopoietic cells (Siden *et al.*, 1981; Levitt and Cooper, 1980), in malignant pre-B cells (Alt *et al.*, 1981; McCune and Fu, 1981; Siden *et al.*, 1979; Perry and Kelley, 1979; Mains and Sibley, 1982; Hendershot and Levitt, 1984; Alt *et al.*, 1982) or in hybridomas obtained by the fusion of fetal liver pre-B cells with myeloma cells (Maki *et al.*, 1980; Perry *et al.*, 1981; Kloppel *et al.*, 1981). These series of studies have led to conflicting results, describing either the intracellular degradation of μ heavy chains (McCune and Fu, 1981; Siden *et al.*, 1979), their expression at the cell surface (Hendershot and Levitt, 1984) or even their secretion by normal fetal pre-B cells (Levitt and Cooper, 1980). With respect to the nature of the μ chains synthesized, analyses of the μ mRNAs and μ heavy chains present in some cell lines has led to the conclusion that μ_s mRNA was the most abundant species transcribed (Perry *et al.*, 1981; Kloppel *et al.*, 1981) whereas in other cell lines μ_s and μ_m RNAs were present in roughly equal amounts (McCune and Fu, 1981; Perry and Kelley, 1979; Mains and Sibley, 1982; Alt *et al.*, 1982).

We have used murine adult bone marrow pre-B cells, since bone marrow is the site of synthesis of B cells in the adult, and very large numbers of new B cells are synthesized daily (Opstelten and Osmond, 1983; Landreth *et al.*, 1981). A cell population containing a majority of pre-B cells uncontaminated by B cells was obtained by a combination of purification steps and used to study the biosynthesis, properties, intracellular pathways and fate of the μ chains, as well as the nature of the mRNA present in these cells.

Results

Purification of bone marrow pre-B cells

The first step in the fractionation of bone marrow cells was a centrifugation on a 5–15% sucrose gradient allowing the selection of medium to small lymphocyte-like cells, which banded near the top of the gradient, while various myeloid cells, plasma cells and most polymorphonuclear cells (PMN) sedimented. Contaminant PMN were then eliminated by adherence to plastic dishes, and B lymphocytes were removed by panning with goat anti-mouse Ig antibodies. To obtain a complete removal of B cells it was still necessary to treat the non-adherent cells with an anti-mouse L chain antibody and complement (C'). The purified cell population contained at least 50% cu^+, sIg^- (pre-B) cells with a negligible contamination by sIg^+ cells (<0.5% of the amount of pre-B cells); this was confirmed by surface radioiodination, which failed to detect any labelled Ig chains (see below) the remaining cells being mostly small to medium round cells, erythroblasts and probably T lymphocytes. This purified population will be referred to below as 'pre-B cells'.

Intracellular immunoglobulin chains observed after biosynthetic radioactive labelling

The Ig chains synthesized by bone marrow pre-B cells after a 1 h pulse with [^{35}S]methionine were analyzed by immunoprecipitation followed by SDS-PAGE, in reduced and non-reduced form. The reduced immunoprecipitate contained both μ and L chains (Figure 1A, lane 1). Thus, in order to discriminate between free and L chain-associated μ chains, all further analyses were performed without reduction. It was thus seen that free μ chains, in mono or dimeric (μ_2) forms

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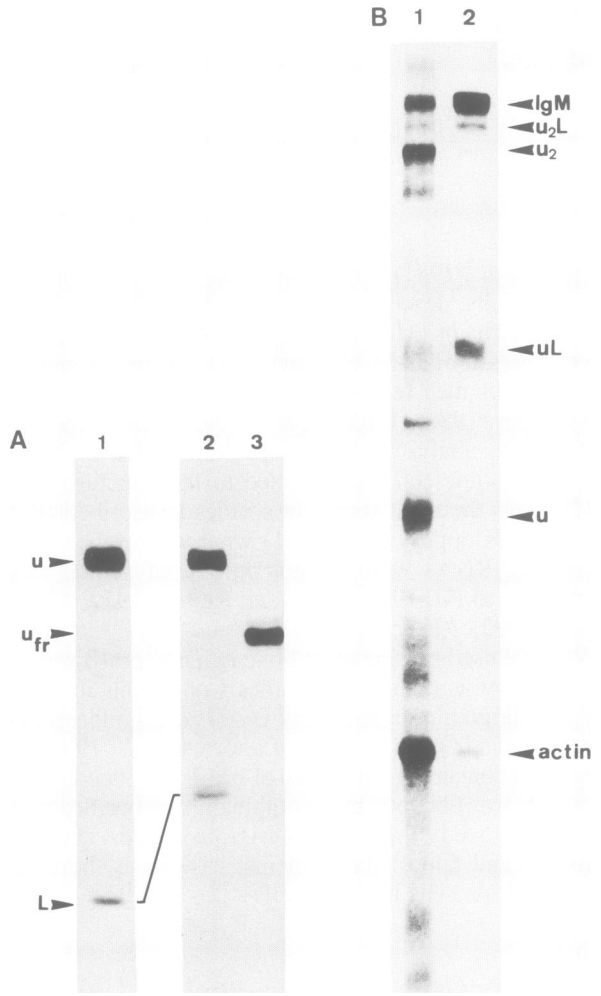

Fig. 1. SDS-PAGE of reduced and non-reduced anti-μ immunoprecipitates from the lysate of biosynthetically-labelled bone marrow cells. **A**: 17.5% SDS-PAGE of reduced anti-μ immunoprecipitates from pre-B cells obtained as described in Materials and methods and lysed in the presence of PMSF, **lane 1**; or from B lymphocytes (**lane 2**) or pre-B cells (**lane 3**), lysed in a medium devoid of PMSF. u = μ heavy chains; μ_{fr} = 48-kd fragmented μ chains; L, light chains. **B**: 7.5% SDS-PAGE of non-reduced anti-μ immunoprecipitates from bone marrow cells labelled for 1 h with [^{35}S]-methionine and lysed with complete anti-proteolytic protection. **Lane 1**, pre-B cell population; **lane 2**, B lymphocytes recovered after panning during the purification of the pre-B cell population. The nature of the different Ig species is indicated on the right side of the gel.

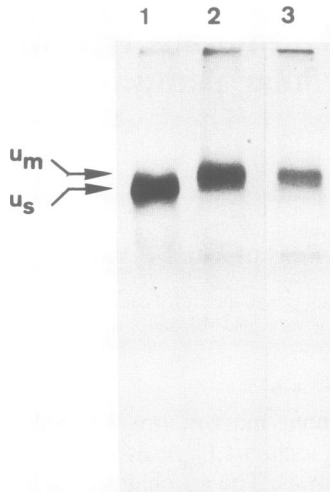

Fig. 2. *In vitro* translation of μ mRNAs. Total RNA isolated by the guanidinium thiocyanate method was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine, μ chains were specifically immunoprecipitated and run on a 17.5% SDS-PAGE. The translation was directed by RNA from: **lane 1**, LPS-induced plasma cells; **lane 2**, sucrose-gradient separated bone marrow cells without further purification (see Materials and methods); **lane 3**, pre-B cells (in this experiment, the last cytotoxic step in the presence of anti-mouse L chains + C′ was omitted). Migration of μ_m and μ_s is indicated on the left side of the gel.

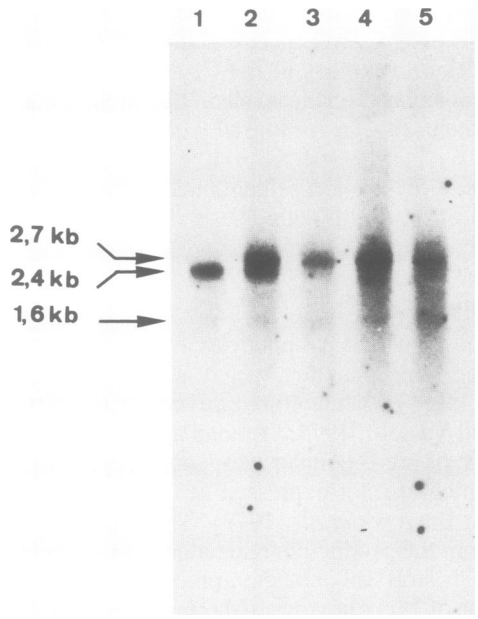

Fig. 3. Northern blot analysis of μ mRNAs. RNAs prepared in Figure 2 were denatured with glyoxal and DMSO, separated on a 1% agarose gel and transferred to nitrocellulose. Specific μ mRNAs were detected by hybridization with a Cμ probe nick-translated to a specific activity of 2 x 10^8 c.p.m./μg. RNAs are from: **1**, LPS-induced plasma cells; **2**, splenic small lymphocytes separated on a sucrose gradient; **3**, bone marrow cells separated only by sucrose gradient; **4**, bone marrow B lymphocytes obtained by panning; **5**, bone marrow pre-B cells. Sizes indicated on the left were determined by reference either to the migration of the 28S and 18S RNAs, run on adjacent lanes, or to the migration of pBR 322 restriction fragments.

were the predominant Ig chains, but IgM monomers and some μL half monomers were also observed in all cases (Figure 1B, lane 1), although in amounts varying somewhat between different experiments. In contrast, B cells selected by panning during the purification procedure never contained free μ chains, but only IgM, mainly as monomers or half monomers (Figure 1B, lane 2).

Analysis of the pre-B cell μ chains mRNA

This analysis was performed in two ways: by translation in a rabbit reticulocyte lysate: pre-B cell mRNA directed the synthesis of slightly larger (μ_m) chains than plasma cells (μ_s) mRNA (Figure 2); and by Northern blot analysis, comparing the mRNA extracted from plasma cells, spleen mature B cells and bone-marrow B and pre-B cells (Figure 3): plasma cells contain only the 2.4-kb μ_s mRNA, spleen B cells both 2.4-kb μ_s and 2.7-kb μ_m mRNAs (in agreement with previous biosynthetic observations: Vassalli *et al.*, 1980) and bone marrow pre-B cells only the 2.7-kb μ_m mRNA. In all preparations, another RNA of ~1.6 kb also hybridized with the μ cDNA probe; its nature is unknown.

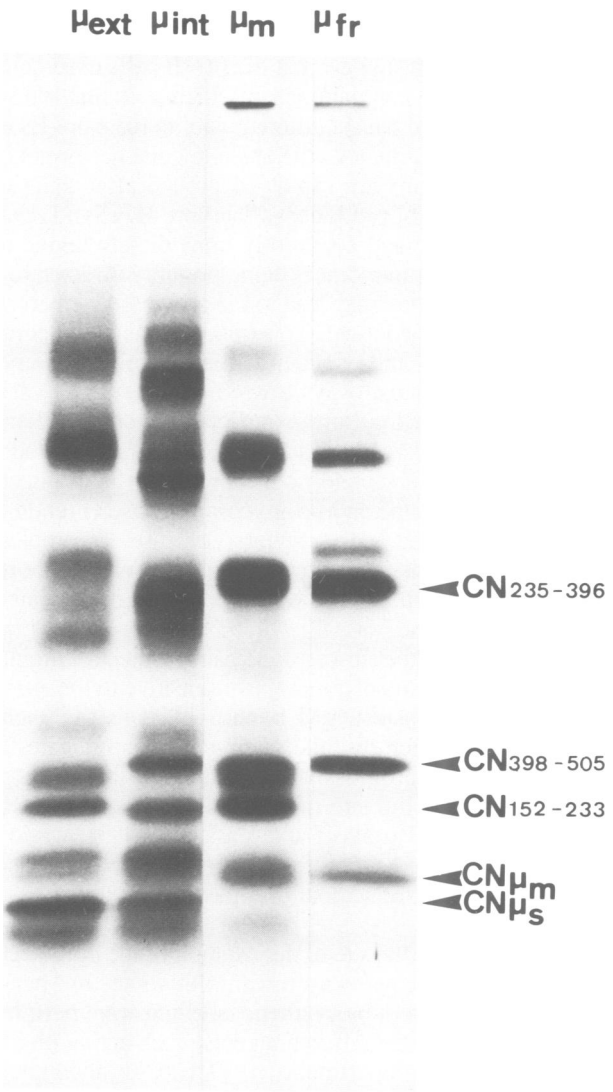

Fig. 4. CNBr peptide mapping of the pre-B cell 48-kd μ chain fragment. CNBr peptides were prepared as described in Materials and methods and separated on 10–18% gradient SDS-PAGE. μ_{ext}:μ_s chains secreted by plasma cells; μ_{int}: intracellular plasma cell μ_s chains; μ_m: B lymphocyte membrane μ_m chains; μ_{fr}: pre-B cell 48-kd μ chain fragment. Identification of the peptides by radioactive sequencing and all other relevant details have been previously reported (Vassalli *et al.*, 1980; Jaton and Vassalli, 1980).

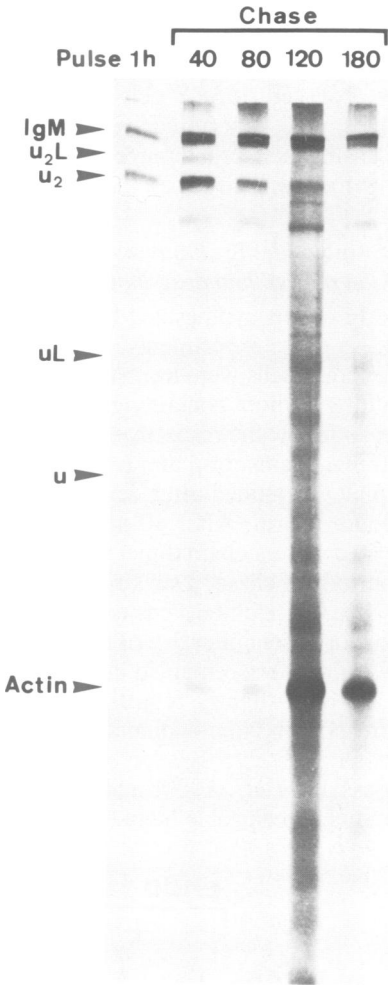

Fig. 5. Pulse-chase labeling of pre-B cells. Pre-B cells were pulsed for 1 h with [^{35}S]methionine or pulsed for 1 h, washed and chased in unlabelled medium for the indicated period of time. The non-reduced immunoprecipitated lysates were run on 7.5% SDS-PAGE.

Difference in proteolytic sensitivity between free and L chain-bound μ chains of pre-B cells

In the course of the biosynthetic experiments described above, it was noted that when the cell lysis was performed in the absence of strong anti-protease inhibitors (see legend of Figure 1), most, but not all, the μ chains were degraded into a specifically immunoprecipitable shorter fragment of ~48 kd. This fragment resulted from the proteolytic cleavage of free μ chains and not of IgM monomers since it was only present in pre-B cells and not in bone marrow B lymphocytes (selected during the panning procedure) (Figure 1A, lane 2 and 3), and since in unreduced gel this fragment was present in mono- or dimeric (96 kd) forms, while μ_2-L_2 monomers or μ-L half monomers were also detected (not shown), indicating that they were the source of the intact μ chains also detected in reduced gels (Figure 1A, lane 3). The precise localization of the proteolytically vulnerable segments of free μ chains was determined by (i) CNBr peptide analysis of the μ chain fragment compared with intact μ chains; (ii) radioactive sequencing of the fragment. Pre-B cells were labelled with [^{35}S]cysteine, the μ chain fragment was eluted from SDS-gels, cleaved by CNBr, then re-analyzed on SDS gels; this type of analysis has been used previously to identify spleen B cell membranous (μ_m) and secretory (μ_s) chains, whose CNBr peptides thus separated had been identified by radioactive sequencing, allowing the recognition of a C-terminal peptide distinct for these two types of chains (Vassalli *et al.*, 1980; Jaton and Vassalli, 1980). The C-terminal μ_m peptide was present in the μ chain fragment, indicating that pre-B cells synthesize only μ_m chains in agreement with the results obtained with μ chain mRNA; the fragment lacked the peptide which corresponds to part of the Cμ_1 domain (residues 152–233) (Figure 4). Since no unusual peptides of small size were observed, this suggests that all the N-terminal part of the free μ chains is degraded to generate the 48-kd fragment. A methionine residue is present in μ chains in position 234, i.e., close to the N-terminal end of the μ_2 domain, and thus the number of Edman degradative cycles performed on the [^{35}S]methionine-labelled 48-kd fragment isolated from SDS-gels should allow the location of the N-terminal end of the fragment. This experiment showed that the N-terminal ends lie three residues before

methionine 234 as well as 11, 13, 18 and 20 residues upstream, i.e., very close to the junction between the $C\mu_1$ and $C\mu_2$ domains; this heterogeneity probably corresponds to the slight size variability observed in SDS gels (Figure 1A). The N-terminal residues thus determined indeed correspond to cleavage sites sensitive to a broad range of proteases including trypsin-like enzymes and elastases (Auffray and Rougeon, 1980a).

Fate and processing of the Ig chains synthesized in bone marrow pre-B cells, in normal and drug-induced altered conditions

The fate of the Ig chains synthesized by pre-B cells was investigated in pulse-chase experiments. After a 1 h pulse with [^{35}S]methionine, pre-B cells were incubated for various periods of time in a culture medium containing an excess of unlabeled methionine. To follow the respective fate of molecules containing either free μ chains, or μ and L associated chains, the immune precipitates obtained after cell lysis were analyzed only in non-reduced form. After 40 min of chase, the main Ig chains observed were μ chain dimers and IgM monomers. After further periods of chase, a striking observation was the progressive disappearance of the μ chain dimers, which became barely detectable or absent after 3 h of chase. In contrast, the amount of IgM monomers remained constant, with the progressive increase of a slightly retarded form of molecules (Figure 5), corresponding to monomers in which the μ chains have undergone terminal glycosylation by the addition of complex sugars (Vassalli *et al.*, 1980, and see below). Neither Ig chains nor immunoprecipitable fragments were ever detected in the culture medium at the end of the chase period (not shown).

These obervations suggest that the pre-B cells used consist of two unequal sub-populations, in which μ chains follow a different pathway and have a different fate: in most pre-B cells, productive rearrangement of the L chain gene has not occurred, and free μ chains are rapidly degraded after their synthesis; in a minority of pre-B cells ('late' pre-B cells), L chains are also synthesized and apparently immediately bound to μ chains, the resulting monomers being possibly directed to the cell surface, where they may lead to the progressive emergence of the sIg^+ B cell phenotype. To explore the sites of degradation of μ_2 molecules and of accumulation of IgM monomers, and the intracellular pathways towards these sites, a variety of pulse-chase or cell culture experiments were performed.

First the Ig chains obtained from cells pulse-labelled for 1 h and chased for a second hour were either kept intact or treated with the enzyme endo-β-glycosaminidase H (endo-H), which removes the core sugars of the incompletely glycosylated μ chains, thus accelerating their electrophoretic migration, but has no effect on completely glycosylated μ chains (Tartakoff and Vassalli, 1979; Vassalli *et al.*, 1980). Figure 6 shows that the slowest IgM band, which accumulates in pulse-chase experiments, remains insensitive to the enzyme endo-H while the fastest IgM band as well as the μ chain dimers are accelerated by this treatment. This indicates that the IgM monomers progressively reach the distal Golgi compartment, which is the site of terminal glycosylation (Roth and Berger, 1982; Tartakoff and Vassalli, 1983), while the μ chain dimers never acquire the terminal sugars and thus are degraded intracellularly without ever passing through the Golgi complex.

To further explore the site of degradation of the μ_2 molecules and the intracellular pathway towards this site, the periods of chase after 60 min of biosynthetic labelling were performed in the presence of three different agents, each acting on a different cellular subcompartment. (i) FCCP, an uncoupler of oxidative phosphorylation, which blocks or retards the transit of newly synthesized proteins from the rough endoplasmic reticulum (RER) to the Golgi, but does not affect the transport through the Golgi, once the Golgi complex has been reached by the proteins in transit (Jamieson and Palade, 1968): this drug delayed μ_2 dimer degradation (Figure 7); it must be noted, however, that the block of exit from the RER was usually not complete, since full glycosylation of IgM monomers was not completely prevented. (ii) Monensin, which interferes with the transit of secretory or membrane proteins through the Golgi complex (Tartakoff and Vassalli, 1977; Griffiths *et al.*, 1983): μ_2 degradation was also retarded, and usually full glycosyla-

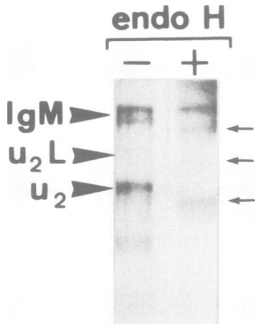

Fig. 6. Sensitivity of the different Ig species to endo-H. Pre-B cells were labelled for 1 h with [^{35}S]methionine, washed and chased in unlabelled medium for 1 h. The immunoprecipitated cell lysate was divided in two, one half was directly suspended in sample buffer, and the other was subjected to endo-H treatment. The analysis is in non-reduced form, on 7.5% SDS-PAGE. Only the upper part of the gel, containing μ dimers and IgMs, is shown. Indication of the position of the endo-H-sensitive molecules is on the right of the gel.

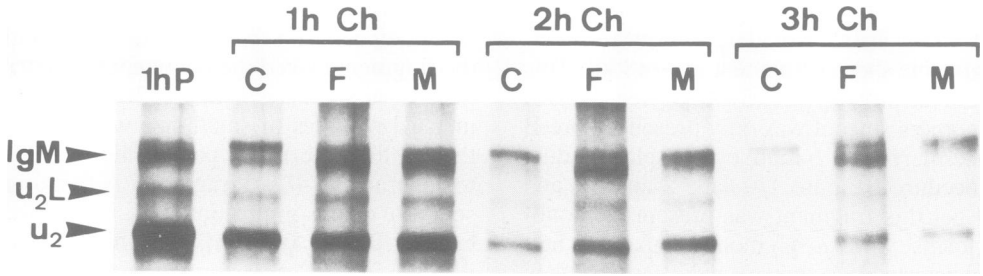

Fig. 7. Pulse-chase of pre-B cells in the presence of FCCP and monensin. Pre-B cells were pulsed and chased as in Figure 2. Chase incubations were performed in control conditions (**C**), in the presence of 10 μM FCCP (**F**) or 1 μM monensin (**M**). Only the upper part of the gel, containing the μ dimers and IgMs, is shown. Identification of the Ig species is on the left of the gel.

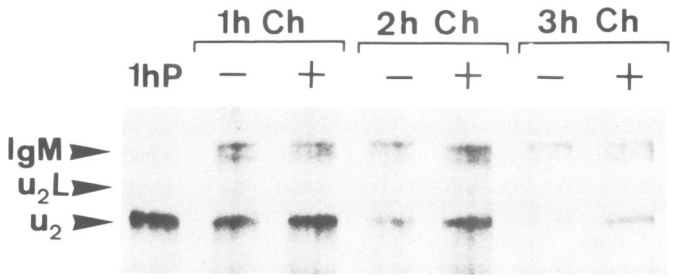

Fig. 8. Pulse chase of pre-B cells in the presence of chloroquine. Pre-B cells were pulsed and chased as in Figure 2. Chase incubations were conducted in control conditions (−), or in the presence of 100 μM chloroquine (+). Only the upper part of the gel, containing the μ dimers and the IgMs, is shown. Identification of the Ig species is on the left of the gel.

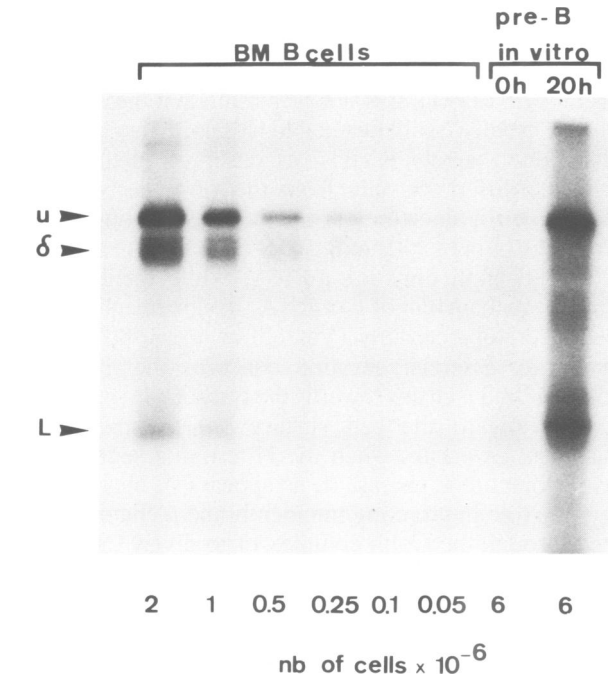

Fig. 9. Detection of surface IgM chains by vectorial radioiodination. Pre-B cells, taken at 0 or 20 h after the initiation of the culture, were labelled by lactoperoxidase-catalyzed radioiodination. Anti-Ig immunoprecipitates were analyzed on reducing 17.5% SDS-PAGE. On the left side are displayed the immunoprecipitates corresponding to graded numbers of similarly labelled bone marrow sIg^+ cells selected during the panning step. Positions of μ, δ, and light chains are indicated on the left of the figure. The number of cells analyzed in each track is indicated at the bottom of the figure.

tion of IgM as well (Figure 7). (iii) Chloroquine, which by raising the lysosomal pH inhibits the function of lysosomes (Wibo and Poole, 1974; Ohkuma and Poole, 1978): μ_2 dimer degradation was retarded, although not completely prevented (Figure 8). It must be pointed out that these observations were consistent throughout repeated experiments. Taken together these results suggest that the μ_2 dimers are catabolized mostly in the lysosomal compartment, which they reach through a monensin-sensitive Golgi subcompartment, without ever reaching the distal part of the Golgi complex since full glycosylation never occurs. In no circumstances during these various pulse-chase experiments was a 48-kb fragment observed.

The terminal glycosylation of IgM monomers observed in the pulse-chase experiments suggest that these molecules may reach the cell surface and progressively accumulate in this location. This was investigated by surface radioiodination: an aliquot of pre-B population was radioiodinated, and the remaining population cultured for various periods of time, followed by radioiodination. Figure 9 shows the anti-mouse Ig immunoprecipitates obtained from radioiodinated pre-B cells before and after a 20 h culture, and, for comparison, from various numbers of bone marrow B cells isolated in the same experiment. While no Ig chains were detected on the pre-B cells labelled before culture, large amounts of μ and L chains were detected at the end of the culture, although in markedly lesser amounts than with comparable numbers of bone marrow B lymphocytes [which, in addition, also bear δ chains (Vitetta *et al.*, 1975; Vitetta and Uhr, 1977)]. Parallel studies using immunofluorescence to detect the appearance of surface Ig on individual pre-B cells showed that after 7 h in culture, ~10%, and after 24 h, ~20−30% cells have detectable surface Ig.

Discussion

The salient findings emerging from the study of Ig chain biosynthesis by adult bone marrow cells will be discussed in the following order: (i) the μ chains synthesized and the μ mRNA detected are only of the μ_m variety, in contrast to what has been reported with pre-B cell lines or tumors; (ii) two varieties of μ chains are observed, free and L chain bound, with different properties (sensitivity to proteolytic enzymes), intracellular pathway and ultimate fate; (iii) does the high proteolytic sensitivity of the N-terminal domain of the free μ chains reflect a difference in conformation between free and L chain-bound μ chains which is essential in directing their respective intracellular pathways and thus ultimate fate?

From the conflicting results obtained with a variety of pre-B cell lines or tumors, probably the most widely held view is that pre-B cells synthesize μ_m and μ_s mRNA in roughly equal amounts; in addition, in most of the cell lines studied, a number of untranslated mRNA of aberrant size, ranging from 1.9−3.0 kb, have also been detected, in amounts comparable with that of 2.4-kb μ_s and 2.7-kb μ_m mRNA (Perry *et al.*, 1981; Perry and Kelley, 1979; Alt *et al.*, 1982). Using mouse fetal liver cells, Levitt and Cooper (1980) reported the secretion of molecules considered as being free μ chains. With the adult murine bone-marrow pre-B cells used in the present experiments, the results were clear cut as judged: (a) by the size of both the mRNA and its cell free translation product, (b) by the pattern of CNBr peptides obtained from biosynthetically labelled μ chains isolated from SDS gels, and (c) by the absence of secretion product. Only μ_m mRNA and μ_m polypeptide chains were detected. This contrasts with spleen B lymphocytes which synthesize both μ_m and μ_s chains (Vassalli *et al.*, 1980) and contain both μ_m and μ_s mRNA. A single additional μ chain cDNA hybridizing RNA of 1.6 kb was present in the different varieties of normal cells of the B lineage, in contrast to the observation made with different cell lines.

Biosynthetic experiments showed that μ_m chains occur in pre-B cells in two forms: the majority is unbound to L chains and assembled in dimers, while a minority is associated with L chains, forming IgM molecules. These two forms of μ_m chains differ dramatically in sensitivity to proteolytic enzymes and intracellular fate.

The exquisite proteolytic sensitivity of the N-terminal do-

mains of free μ chains was fortuitously revealed in experiments in which cell lysis, after biosynthetic labelling, was performed in the absence of proteolytic enzyme inhibitors: free μ_m chains were transformed into a 48-kd fragment, with no other detectable degradation product, while L chain-bound μ_m chains were intact. That this is a general property of free μ chains, not restricted to pre-B cell free μ_m chains, was observed in subjecting purified biosynthetically labelled μ_s chains and secretory IgM monomers to a short treatment by small concentration of trypsin or elastase: a 48-kd fragment is observed with free μ chains, in the absence of any other short fragments, while μ chains present in IgM monomers remain intact (Thorens and Vassalli, unpublished observations). In the present experiments CNBr peptide analysis and radioactive sequencing of the 48-kd pre-B cell μ_m chain fragment indicate that it results from extensive proteolysis of the entire $C\mu_1$ and V_H domains and represents the intact C-terminal part of the molecule.

The intracellular fate of free or bound pre-B cell μ chain is also entirely different since the free chains undergo rapid intracellular degradation while the bound IgM cells are directed to the cell surface. The inability of free μ chains to reach the plasma membrane has been well documented (McCune and Fu, 1981; Mains and Sibley, 1982, 1983), although exceptions have been observed in the case of some malignant cells (Hendershot and Levitt, 1984). The present study shows that free μ chains indeed turn over intracellularly in normal pre-B cells. The combined use of enzymes and drugs interfering with a given cell function, allows us to conclude that degradation occurs mainly because the free μ chains are directed towards the lysosomal compartment since, during a chase period after pulse-labelling, use of FCCP also retards μ chain degradation. Therefore, μ chains must leave the RER in order for complete degradation to occur. Secondly monensin also retards degradation. Thus, free μ chains must pass through at least part of the Golgi complex to undergo degradation. The μ_2 chain dimers always remain sensitive to endo-H (in contrast to the IgM monomers): since this enzyme is unable to remove the oligosaccharide side chains of μ molecules once terminal glycosylation has occurred (Tartakoff and Vassalli, 1979; Vassalli *et al.*, 1980), and since terminal glycosylation occurs in the distal part of the Golgi complex (Roth and Berger, 1982; Tartakoff and Vassalli, 1983), free μ chains never reach the '*trans* Golgi'. Chloroquine also retards free μ chain degradation; thus, it appears that free μ chains are diverted from the proximal part of the Golgi complex to the lysosomal compartment, where they are fully degraded. It is possible that this monensin-inhibited, lysosome-directed pathway corresponds to the recently described pathway followed by the lysosomal enzymes, which goes through the *cis* but avoids the *trans* Golgi compartment (Brown and Farquhar, 1984). The failure to detect a 48-kd fragment along this pathway indicates that this type of cleavage (if it does occur *in vivo*), can only be a late event, in which case the half-life of this fragment could be so short as to make it undetectable.

The L chain bound μ chains present as monomeric IgM molecules within the bone-marrow pre-B cells do not result from a small contamination by B cells, since surface IgM molecules were not detected by immunofluoresence or by surface radioiodination techniques. In contrast to free μ chains, IgM monomers go through the distal part of the Golgi apparatus, since terminal glycosylation was observed, and they appear progressively in increasing amounts on the membrane of a number of cells, as detected after a few hours in culture by immunofluorescence and surface radioiodination; the progressive surface expression of IgM monomers by these cells represents a transition from the pre-B to the B lymphocyte phenotype. Thus, the cell synthesizing intracellular IgMs most probably represent a 'late' pre-B cell stage in which light chain genes have been productively rearranged, as opposed to the 'early' pre-B cells which synthesize only μ chains and in which probably only the μ chain genes have undergone productive rearrangement. The occurrence of pre-B sIg^- cells containing cytoplasmic IgM detectable by immunofluorescence has been reported in man and rabbit (Kubagawa *et al.*, 1982; Gathings *et al.*, 1982). The percentage of these 'late pre-B cells', compared with the cells synthesizing only μ chains, may have been somewhat increased in the pre-B cell population studied here, since the sucrose gradient used selects for cells of small size, thus eliminating the large pre-B cells, which have rearranged only their heavy chain genes (Coffman and Weissman, 1983). However, in view of the large daily output of new B cells in bone marrow (Opstelten and Osmond, 1983; Landreth *et al.*, 1981), it is not surprising that these late pre-B cells are usually easily detectable. It might also be pointed out that bone marrow B cells, as observed in the present experiment, differ not only from the very 'early B cells' obtained *in vitro*, since they bear not only IgM but IgD, but also from the bulk of splenic B lymphocytes of the same size, since they contain only μ_m mRNA, while the latter also contain an equal amount of μ_s mRNA, in agreement with their detected secretory capacity (Vassalli *et al.*, 1980).

Finally, an essential question concerns the mechanism directing the free μ chains towards the lysosomal compartment instead of toward the cell surface *via* the *trans* Golgi compartment as occurs when the N-terminal segment of μ chains is bound to L chains. If, as appears, L chain binding plays a key role in targeting the membrane μ chain traffic to proceed through the Golgi complex or to divert towards the lysosomes, how does it operate? The high proteolytic vulnerability of the $C\mu_1$ and V_H domains of free μ chains might not simply reflect the unmasking of a few proteolytic sites inaccessible to enzymatic action when this part of the molecule is associated with L chains. Since no fragments other than the 48-kd C-terminal fragment are detected, as mentioned above, it appears that the whole N-terminal fragment is digested, even though it contains the $C\mu_1$ domain, structurally very comparable with the other three $C\mu$ domains which are left entirely intact. Thus, this very high proteolytic sensitivity of the V_H and $C\mu_1$ domain may reflect a very unstable three-dimensional conformation of this region of the μ chain molecule, requiring L chain association to become stabilized. This situation appears to be analogous to that of another two-chain membrane molecule, which has a structural similarity with the immunoglobulin chains, namely the HLA molecule, made of a membrane HLA heavy chain, whose N-terminal segment is associated with a β_2 microglobulin chain; when they are not bound to β_2 microglobulin, HLA-A heavy chains lose half of their characteristic β-pleated sheets (Lancet *et al.*, 1979). The possibility should be considered that it is this very instability of N-terminal domains of free μ chains which is instrumental in targeting them towards the lysosomes. In the absence of β_2 microglobulin synthesis, HLA heavy chains also fail to be expressed at the cell surface because they undergo intracellular degradation (Ploegh *et al.*, 1979); although this intracellular pathway has not been explored, it is tempting

to think that it may not be different from that from free μ_m chains. There is also evidence that a modified tertiary structure interferes with the proper intracellulular targeting and pathway of other proteins, such as influenza virus hemagglutinin (McQueen *et al.*, 1984), Sindbis Forest virus E1 glycoprotein (Arias *et al.*, 1983) and defective collagen (Berg *et al.*, 1980, 1984). Another striking observation also consistent with this hypothesis is that free immunoglobulin heavy chains, in contrast to free L chains, have never been found to be secreted by plasma cells, except in the case of the various forms of heavy chain diseases (Seligmann *et al.*, 1979). In these conditions, malignant plasma cells release free heavy chains which are characterized by extensive deletion in their N-terminal domains, thus potentially depriving these chains of the region of conformational instability which may target them towards the lysosomal pathway.

Materials and methods

Animals and cell cultures

6–8 week-old mice of a variety of strains: (C57/Bl6 x BALB/c)F_1, (C57/Bl6 x T6T6)F_1, C3H, CBA/Ca, BALB/c or Swiss mice, were used throughout this work. Bone marrow cells were flushed out with Hank's balanced salt solution (HBSS) from femurs, tibias and humeri, dispersed by repeated passages through a 20-gauge needle, washed twice in HBSS and the red cells lysed in 0.75% NH_4Cl, 20 mM Tris-HCl pH 7.4. Sucrose gradient separation was performed on linear 5–15% gradients in 50% HBSS containing 20% newborn calf serum (Ryser and Vassalli, 1974). PMN adherence was peformed on tissue culture plastic dishes in Dulbecco's modified Eagles medium (DMEM), for 20 min at 37°C. Removal of the B lymphocytes by panning was according to Wysocki and Sato (1978) using affinity purified goat anti-mouse immunoglobulin antibodies. For complement treatment, cells were suspended at 3 x 10^6 cell/100 μl of HBSS, and incubated with 1/5 dilution of an anti-mouse light chain serum (raised against purified normal mouse IgG-derived light chains), for 30 min at 0°C. Complement (Rabbit, low tox M, Cedarlane, Hornby, Ontario) was then added at a 1/10 dilution and cells incubated 30 min at 37°C, in the presence of 0.03% NaN_3. Dead cells were removed by centrifugation on Ficoll-Hypaque (Davidson and Parish, 1975). Cultures of unlabelled cells were performed in DMEM containing 10% fetal calf serum (Gibco), 2 mM glutamine, 5 x 10^{-5} M β-ME, penicillin 100 mU/ml and streptomycin 100 μg/ml. IgM plasmablasts were obtained by cultures of spleen cells in the presence of 50 μg/ml LPS, as described (Tartakoff and Vassalli, 1979).

Cell labelling and immunoprecipitation

Biosynthetic labelling and pulse-chase experiments were performed with [^{35}S]-methionine or [^{35}S]cysteine, as described (Tartakoff and Vassalli, 1979). When inhibitors were used in the chase, chloroquine (Sigma Co., St. Louis, MO) was at 100 μM, monensin (Calbiochem, La Jolla, CA) at 1 μM and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (DuPont) at 10 μM. Incubations were terminated by sedimenting the cells 5 min at 1000 *g*, washing them twice in HBSS and lysing them in lysis buffer: 50 mM Tris HCl pH 7.4; 25 mM KCl; 5 mM $MgCl_2$ containing 0.5% NP-40 (Fluka, Buchs, Switzerland); 1 mM phenylmethylsulfonyl fluoride (PMSF) (Merck, Darmstadt, FRG), 1 mM tosyl-arginine methyl ester (Sigma Co., St-Louis, MO), 1 μM tosyl-lysine-chloromethane (Boehringer, Mannheim, FRG), 10 mM iodoacetamide (Fluka, Buchs, Switzerland). Lysates were cleared by centrifugation at 100 000 *g* for 30 min.

Surface radioiodination was performed as described with ^{125}I (Pink and Ziegler, 1979). After removal of the nuclei by low speed centrifugation, the cell lysate was passed on a small Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column and the excluded material was spun at 100 000 r.p.m. for 30 min.

Immunoprecipitations with specific rabbit anti-mouse μ chains or anti-mouse immunoglobulin antibodies, followed by fixed *Staphylococcus aureus*, and SDS-PAGE of the immunoprecipitates were performed and analyzed by fluorography as previously described (Tartakoff and Vassalli, 1979).

For endoglycosaminidase H (Endo-H) (Seikagaku Kogyo, Tokyo, Japan) treatment, the immunoprecipitate was resuspended in 0.3 M Na acetate pH 5.5 containing 1% SDS, boiled for 5 min, centrifuged and the supernatant precipitated with acetone. The precipitate was suspended in 50 mM Na acetate pH 5.5 containing 0.1% SDS and 1.5 mU of Endo-H. Incubation was for 24 h at 37°C, with one addition of the same amount of enzyme after 15 h.

Peptide mapping and sequencing

[^{35}S]cysteine-labelled proteins were extracted from SDS-PAGE slab gels, cleaved with CNBr and peptides analyzed on 10–18% gradient SDS-PAGE as described (Jaton and Vassalli, 1980; Vassalli *et al.*, 1980).

[^{35}S]Methionine-labelled proteins were isolated from SDS-PAGE and submitted to Edman's degradation in a Beckman sequencer, as previously described (Jaton and Vassalli, 1980).

Preparation of RNA, cell-free translation and Northern blots

Cells were lysed in guanidinium thiocyanate 5 M, 0.5% lauryl sarkosin, 50 mM Na citrate pH 7.0. CsCl was added to the lysate at 1 g/2.5 ml of lysate. The lysate was loaded on a 5.7 M CsCl solution containing 0.1 M EDTA and centrifuged in a SW-41 rotor at 36 000 r.p.m. for 18 h, at 20°C (Chirgwin *et al.*, 1979). The RNA collected at the bottom of the tube was precipitated twice with ethanol and resuspended in water. Rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (1976) and used as described by Auffray and Rougeon (1980b).

For Northern blots, RNA was denatured with glyoxal 1 M in phosphate buffer pH 7.0 for 1 h at 50°C as described by McMaster and Carmichael (1977), and separated on 1% horizontal agarose gel. RNA was transferred to nitrocellulose (Schleicher and Schuell), dried and baked as described by Thomas (1980). Nick translation of pμ-183 (kind gift of F. Rougeon, Institut Pasteur, Paris) (Auffray and Rougeon, 1980a) was performed with a NEN nick translation kit, to specific activity of 2–5 x 10^8 c.p.m./μg. Pre-hybridization was performed in 4 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.0), 5 x Denhardt (1 x Denhardt: 0.2 g/l bovine serum albumin, 0.2 g/l polyvinylpyrrolidone, 0.2 g/l Ficoll), 0.1% SDS, for 3–12 h at 65°C. Hybridization was in 4 x SSC, 5 x Denhardt, 0.1% SDS, 50 mM phosphate buffer pH 7.0 at 65°C for 36–48 h. Washing of the blots was for 10 min twice with 2 x SSC, 0.1% SDS at room temperature and again for 10 min twice with 0.1 x SSC, 0.1% SDS at room temperature. The dried gels were exposed at −70°C, using a Cronex (DuPont) intensifier screen.

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