The T3/T cell receptor complex: antigenic distinction between the two 20-kd T3 (T3- δ and T3- ϵ) subunits

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Clonally distributed (clonotypic) antigen receptors on human T lymphocytes (α and β chains) are associated with three invariable T3 polypeptide chains (T3 γ , δ and ϵ), together forming the T3/T cell receptor complex. Monoclonal antibodies prepared against the two 20-kd T3 polypeptide chains demonstrated that T3- δ and T3- ϵ are distinct polypeptide chains. Only one monoclonal antibody (anti-T3- δ chain) reacted with the T cell surface as judged by indirect immunofluorescence, and by its mitogenicity for quiescent peripheral blood lymphocytes. Immunohistological staining and immunoprecipitation experiments showed that both the T3- δ and T3- ϵ chains are T cell-specific. As seen with the anti- α/β chain reagent WT-31, anti-T3- δ chain monoclonal antibodies stained medullary thymocytes more intensely than cortical thymocytes, whereas the difference between the staining of cortical and medullary thymocytes was generally not apparent with anti-T3- ϵ chain antibodies. Because of this specificity and their ability to react with both the denatured and the native forms of each polypeptide chain, these new monoclonal reagents will be useful tools in studies of the biosynthesis and cell surface expression of the T3/T cell receptor complex during normal and malignant thymic differentiation.

Key words: T3 complex/T cell receptors/monoclonal antibodies/thymic differentiation/sequential Western blotting

Introduction

T lymphocytes play a central role in the immune response. Cytotoxic T lymphocytes eliminate tumor cells, virally infected cells and are involved in the rejection of grafted tissues. Other sets of T lymphocytes (T helper and T suppressor cells) control humoral and cellular responses to foreign antigens. Like B lymphocytes, T cells express specific receptors for antigen on their surface. Recently, it has been found that in humans and mice these T cell receptors are sulfhydryl-linked heterodimers, which consist of two glycosylated polypeptide chains (α and β chains) ranging in mol. wt. from ~37–50 kd (Kappler *et al.*, 1983; Samuelson *et al.*, 1983; Meuer *et al.*, 1983a). In addition, genes encoding the α and β chains have been isolated (Yanagi *et al.*, 1984; Hedrick *et al.*, 1984a, 1984b; Saito *et al.*, 1984). On the surface of human lymphocytes the clone specific (clonotypic) α and β chains of the T cell receptor are associated with the three invariable T3 proteins, thus forming the so called T3/T cell receptor complex (Borst *et al.*, 1983a; Oettgen *et al.*, 1984; Meuer *et al.*, 1983b). These T3 polypeptide chains have mol. wts. of 25 kd (T3- γ chain), and 20 kd (T3- δ and ϵ -chains). The T3- γ and T3- δ

chains are glycoproteins whereas the T3 ϵ -chain does not contain any detectable oligosaccharides (Borst *et al.*, 1982, 1983a, 1983b; Van Agthoven *et al.*, 1981; Kanellopoulos *et al.*, 1983).

Thus far only preliminary evidence has been reported which shows that the T3- δ and T3- ϵ chains are indeed distinct polypeptide chains (Borst *et al.*, 1983a). The non-glycosylated T3- ϵ chain can be labeled by the hydrophobic reagent [¹²⁵I]-iodonaphthylazide, and is not susceptible to Endoglycosidase-H or Endoglycosidase-F (Borst *et al.*, 1983b). In contrast, the T3- δ chain contains N-linked oligosaccharides on a 14-kd polypeptide chain backbone. A 16-kd intermediate digestion product has also been observed during deglycosylation experiments (Borst *et al.*, 1983a).

Several monoclonal antibodies reacting with the human T cell-specific antigen T3 have been described in the past by different groups (Kung *et al.*, 1979; Ledbetter *et al.*, 1981; Beverley *et al.*, 1981; Spits *et al.*, 1983; Tax *et al.*, 1983). Im-

Fig. 1. Immuno dot-blot screening. Denatured T3 proteins (10 ng) were coupled to CNBr-activated paper. Squares of this paper (2 x 2 mm) were incubated with supernatant fluid of hybridoma cells, followed by [¹²⁵I]-sheep F(ab') anti-mouse-IgG. After several washes paper was dried and examined by autoradiography (see Materials and methods). A representative 16 sample analysis is shown. Monoclonals which scored positive are indicated by arrows.

Table I. Characteristics of monoclonal antibodies and rabbit antiserum directed at the 20 kd T3 chains

Antibody	Immuno-globulin isotype	Indirect ^a immunofluorescence	Mitogenicity	Specificity in Western blots
SP-19	IgG1	—	—	T3- δ
SP-20	IgG1	—	—	T3- δ
SP-64	IgG1	—	—	T3- δ
SP-78	IgG1	—	—	T3- δ
SP-34	IgG ₃	+	+	T3- δ
H-89	—	—	—	T3- δ
SP-6	IgG1	—	—	T3- ϵ
SP-10	IgG1	—	—	T3- ϵ

^aDetermined on human peripheral blood lymphocytes, thymocytes and the T-leukemic cell line HPB-ALL.

munoprecipitation experiments have indicated that anti-Leu-4 may be directed at one of the 20-kd T3 components (Borst *et al.*, 1983a). It appears that some of the other previously described monoclonal antibodies (OKT3, UCHT-1) recognize the same set of surface antigens. In an attempt to define better the relationship between the T3 polypeptide chains, we have raised monoclonal antibodies against the purified 20-kd component of the T3 complex. A number of monoclonal antibodies were isolated and characterized and we describe here those that are specific for either one of the two 20-kd subunits of the T3 complex, as indicated by immunoblotting studies. In immunoprecipitation experiments and immunohistological studies we demonstrate that both T3- δ and T3- ϵ are T cell specific.

Results

Antibody reagents directed at the T3 proteins

The proteins of the T3-complex were isolated from large bat-

Fig. 2. Immunoblotting analysis of T3 proteins separated by SDS-PAGE. Partially purified T3 proteins were separated by SDS-PAGE in a minigel system (Materials and methods). After transfer to nitrocellulose membranes, strips were cut which were incubated with various antibodies. Strips were developed with [¹²⁵I]sheep-F(ab') anti-mouse IgG. After several washes radioactive antibodies were visualized by autoradiography. (A) Untreated T3 proteins: lane A, ¹⁴C-labeled mol. wt. markers are phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (48 000), aldolase (29 000) and cytochrome c (14 000); lane B, normal mouse serum; lane C, normal rabbit serum; lane D, rabbit anti-T3- δ (H89); lane E, SP-6; lane F, SP-10; lane G, SP-34; lane H, SP-64; lane I, SP-78. (B) Endo-F-treated T3 proteins: lane A, ¹⁴C-labeled mol. wt. markers; lane B, normal mouse serum; lane C, normal rabbit serum; lane D, rabbit anti-T3- δ (H89); lane E, SP-6; lane F, SP-10; lane G, SP-34; lane H, SP-64; lane I, SP-78.

Fig. 3. Sequential Western blotting analysis of T3- δ and T3- ϵ chains separated by two-dimensional gel electrophoresis. T3 proteins were separated by two-dimensional gel electrophoresis and transferred to a nitrocellulose filter. The filter was developed first with the monoclonal antibody SP-64 and [125 I]sheep-F(ab') anti-mouse IgG (A). After washing the filter at high pH (see Materials and methods), it was re-developed with monoclonal antibody SP-6 and [125 I]sheep-F(ab') anti-mouse IgG (B). The radioactive antibodies were visualized by autoradiography.

ches (30×10^6) of HPB-ALL cells by immunoadsorbent chromatography on an anti-Leu-4-Sepharose column (see Materials and methods). After denaturation in 0.1% SDS, quantities of $\sim 10 \mu\text{g}$ of T3 proteins were injected into a BALB/c mouse at 3 week intervals. After the fourth injection, anti-T3 antibodies were detected in the serum by a dot-blot assay for binding to denatured T3 proteins (see Materials and methods) and by immunoprecipitation. At this time, a last booster injection was given and the mouse splenocytes were isolated 3 days afterwards. Following somatic cell hybridization, cells were seeded into 240 wells. Three weeks later, growth of hybridomas was found in 125 wells, 19 of which secreted anti-T3 antibodies, as judged by a dot-blotting assay (Figure 1). These antibody secreting cells were cloned three times by limiting dilution and were further characterized. All 19 monoclonal antibodies reacted with one of the two 20-kD T3 components (T3- δ or T3- ϵ). In this paper we describe seven of the monoclonals which react with either

T3- δ or T3- ϵ . Their properties are summarized in Table I. Only one monoclonal antibody (SP-34) reacted with T cell surface as judged by indirect immunofluorescence and its ability to stimulate [^3H]thymidine uptake in quiescent peripheral blood T lymphocytes.

Rabbit antisera directed at the glycosylated 20-kD T3 chain (T3- δ) employing a synthetic peptide were prepared. This peptide Phe-Lys-Ile-Pro-Ile-Glu-Glu-Leu-Glu-Asp-Arg-Val-Phe-Val-Cys, represented the 14 N-terminal amino acids determined by sequence analysis (Borst *et al.*, 1984) plus an extra L-cysteine that had been added for coupling purposes. This peptide was coupled to keyhole limpet haemocyanin (KLH) and injected into rabbits. After three injections at 2 week intervals serum was found to react with peptide and denatured T3 proteins in a dot-blot assay (see Materials and methods).

Analysis by Western blotting of anti-T3- δ and anti-T3- ϵ monoclonal antibodies

Since the anti-20-kD T3 monoclonal antibodies had been generated and analyzed with denatured T3 proteins, their specificity was further examined using Western blotting. As shown in Figure 2A, all seven monoclonal reagents and the anti-T3- δ -peptide antiserum were able to detect a 20-kD band on a nitrocellulose filter prepared by transfer of T3 proteins from a SDS gel. Next the reactivity with the two 20-kD T3 chains was investigated. Purified T3 preparations were digested with Endoglycosidase-F and subjected to SDS-PAGE. The extent of digestion was determined using as a positive control the rabbit anti-T3- δ antiserum. As shown in Figure 2B, this rabbit antiserum (H-89) reacted primarily with 14-kD material and relatively weakly with 20-kD protein. This demonstrated that most of the glycosylated 20-kD T3 chain (T3- δ) had been deglycosylated, leaving non-glycosylated protein as the major component of the remaining 20-kD material. As judged by Coomassie Brilliant Blue staining, approximately half of the protein had been converted into a 14-kD band (data not shown). Developing the nitrocellulose filter blotted from the microslab gel of the Endo-F treated material showed that two monoclonals (SP-6 and SP-10) reacted with the remaining 20-kD material, whereas the other five (SP-19, 20, 34, 64 and 78) detected the 14-kD T3 (T3- δ -chain). As expected, conventional anti-T3 reagents (OKT3, anti-Leu-4 and UCHT1) did not react in this type of immunoblotting experiment. Antibody SP-34 is of special interest for it reacted with 20-kD material before Endo-F digestion, but did not react with any Endo-F-treated material (Figure 2). Since T3- δ is the only Endo-F-sensitive polypeptide chain, SP-34 must react with T3- δ .

To show that SP-6 and SP-64 indeed reacted with two different proteins, a sequential Western blotting experiment was conducted after separation of the T3 polypeptides by two-dimensional gel electrophoresis. In this experiment the nitrocellulose filter was first developed with SP-64, then the bound antibody was removed with a high pH wash and the filter was newly developed with SP-6. As shown in Figure 3, panel A the monoclonal antibody SP-64 detected a series of spots which are similar in arrangement to the 20-kD T3 pattern on 2-D gels after immunoprecipitation of cell surface radioiodinated material with anti-T3 reagents (Borst *et al.*, 1982). In contrast, the antibody SP-6 (Figure 3, panel B) detected a smear of 20-kD material which in isoelectric focussing overlapped in part with the protein detected by SP-64. The latter was expected because when the non-glycosylated 20-kD T3- ϵ chain was labeled with [125 I]iodonaphthylazide and was

Fig. 4. Analysis of SDS-PAGE of immunoprecipitates. Detergent lysates were prepared from ^{125}I -labeled HPB-ALL cells (lanes A–D), from ^{35}S -labeled HPB-ALL cells (lane E and H), ^{35}S -labeled Raji cells (lane F) or ^{35}S -labeled Hep-G2 cells (lane G). Immunoprecipitations were done as described previously (Materials and methods) and were analyzed by gradient SDS-PAGE (5–15% in acrylamide) and autoradiography. Lane A, antibody SP-19, NP-40 lysate; lane B, antibody SP-6, NP-40 lysate; lane C, antibody SP-19, NP-40 and SDS lysate; lane D, antibody SP-6, NP-40 and SDS lysate; lane E, antibody SP-6, NP-40 lysate; lane F, antibody SP-6, NP-40 lysate; lane G, antibody SP-64, NP-40 lysate; lane H, antibody SP-64, NP-40 lysate.

compared with the other 20-kD T3 chain (T3- δ) by isoelectric focusing the patterns partially coincided (Borst *et al.*, 1983b).

Taken together, these Western blotting experiments showed that the seven monoclonals detect either the non-glycosylated 20-kD T3 chain (SP-6 and SP-10) or the glycosylated 20-kD T3- δ chain (SP-19, 20, 34, 64 and 78). No antibody has been found as yet that reacts with both chains.

Immunoprecipitation studies

All the selected antibodies were then tested for their ability to precipitate T3 from detergent lysates of radiolabeled HPB-ALL cells. Since the antibodies had been raised against denatured T3 protein, all the immunoprecipitation experiments were carried out both in native and denaturing conditions. A representative SDS-PAGE analysis of such an immunoprecipitate is shown in Figure 4 (lane A–D). The complete results are summarized in Table II. Whereas all experiments were done with radiolabeled HPB-ALL cells, controls were carried out with metabolically labeled Raji cells (a human B lymphoblastoid cell line) and Hep-G2 cells (a human hepatoma cell line) (Figure 4, lanes F and G). These non-T-cells did not contain any proteins which could be identified as T3- δ or T3- ϵ (Figure 4, lanes E and H). This suggested that, like the conventional anti-T3 reagents, the new anti-T3- δ and T3- ϵ monoclonals were T cell specific. This was further investigated by immunohistological staining experiments.

Table II. Summary of the immunoprecipitation experiments with anti-20 kD T3 monoclonals

Monoclonal antibody	Target ^a structure	Lysates from cell surface radioiodinated HPB-ALL cells		Lysates from [^{35}S]-methionine labeled HPB-ALL cells	
		With SDS ^b	Without SDS	With SDS ^b	Without SDS
SP-19	T3- δ	+	–	+	+
SP-20	T3- δ	–	–	–	–
SP-34	T3- δ	+	+	+	+
SP-64	T3- δ	+	+	+	+
SP-78	T3- δ	+	+	+	+
SP-6	T3- ϵ	+	+	–	+
SP-10	T3- ϵ	–	–	+	–

^aDetermined in Western blotting experiments.

^bLysates in NP-40 were boiled for 5 min in the presence of 0.1 %SDS before immunoprecipitation.

Both T3- δ and T3- ϵ are T cell-specific proteins

To test the tissue specificity of the anti-T3- δ and anti-T3- ϵ monoclonal reagents, frozen tissue sections of human thymus, lymph nodes and tonsils were stained using a sensitive indirect immunoperoxidase technique (Cerf-Bensusan *et al.*, 1983). This procedure includes fixation of tissues with acetone before antibody is added. Since the antibodies were made against denatured T3 proteins, and as fixation can lead

Fig. 5. Immunoperoxidase staining of lymph nodes. Sections of lymph nodes stained with SP-64 (A) and SP-6 (B) antibodies. Most of the cells in the paracortex (pc) show intense peripheral staining. In addition, many stained cells are present in the secondary follicle (sf). The staining with SP-64 is more intense than with SP-6. Immunoperoxidase; magnification 160 x.

to denaturation of proteins, it seemed plausible that even those antibodies which did not stain intact cells in an indirect immunofluorescence assay (Table I) could detect their target antigen in this immunohistological procedure. Furthermore, intracellular proteins can also be detected in tissue sections. Indeed, antibodies SP-6, SP-10 and SP-64 stained cells in the human thymus, tonsils and lymph nodes. Selected examples of staining patterns are shown in Figures 5 and 6.

Most of the cells in the paracortical area (T cell area) of the lymph node as well as in the interfollicular area of the tonsils stained with SP-6, SP-10 and SP-64 antibodies (Figure 5A and B). In addition many cells in the secondary follicle stained with both antibodies. The staining with SP-64 was generally more intense than with SP-6. Thus the staining pattern obtained with both antibodies was indistinguishable from that

observed with OKT3 or anti-Leu-4 antibodies (Poppema *et al.*, 1981; Bhan *et al.*, 1981).

To investigate the expression of the target antigens for SP-6 and SP-64 on thymocytes, frozen tissue sections of human thymuses were stained using an immunoperoxidase technique. Since the T3- δ and T3- ϵ chain are part of the T cell receptor complex, a recently generated monoclonal antibody WT-31 that reacts with a common epitope on the T cell receptor α or β chain (Tax *et al.*, 1984; Oettgen *et al.*, 1984), was included in these studies. SP-64 and WT-31 antibodies showed a staining pattern similar to that observed with anti-T3 (OKT3) antibodies (Bhan *et al.*, 1980). The medullary thymocytes stained intensely with SP-64 antibody, and less intensely with WT-31 antibody (Figure 6A and B). In contrast, the staining of cortical thymocytes was faint with both SP-64 and WT-31; however,

Fig. 6. Immunoperoxidase staining of thymus. Sections of thymus stained with SP-64 (A), WT-31 (B) and SP-10 (C) antibodies. SP-64 intensely stains the medullary thymocytes, and faintly stains most of the cortical thymocytes. In addition, a small number of thymocytes scattered in the cortex are also intensely stained. Similar, but less intense staining of medullary and cortical thymocytes is seen with WT-31. The differences in intensity of staining between cortical and medullary thymocytes is not apparent in the section stained with SP-10 antibody. Immunoperoxidase; magnification 160 x.

a small number of thymocytes scattered throughout the cortex stained intensely. This striking difference in the staining of cortical and medullary thymocytes was generally not apparent in the sections stained with SP-6 and SP-10 antibodies due to less intense staining of medullary thymocytes and possibly due to a slight increased staining of cortical thymocytes (Figure 6).

These results strongly suggest that T3- δ and T3- ϵ are T cell specific. Furthermore, expression of the T3- δ polypeptide chain coincides with that of the T cell receptor heterodimer.

Discussion

The monoclonal antibodies that are described here are useful tools for dissection of the complexity of the T cell receptor/T3 complex. Two monoclonals (SP-6 and SP-10) are directed at the T3- ϵ chain. This is the first positive identification of that non-glycosylated 20-kd protein, which does not use a hydrophobic labeling reagent (Borst *et al.*, 1983b). Five other monoclonal antibodies were specific for the glycosylated 20-kd T3- δ chain. Only one of these antibodies (SP-34) detected an epitope which was readily available on

the cell surface. That was not too surprising, because the antibodies were generated and selected with T3 proteins that had been denatured in SDS. SP-34 appears to react with the T3- δ chain only when the oligosaccharide side chains are present.

Western blotting after Endo-F treatment, in addition to two-dimensional sequential Western blots, revealed that none of the antibodies described here reacted with both polypeptide chains. This finding indicated that the two polypeptide chains (T3- δ and T3- ϵ) do not have common antigenic determinants, and is in agreement with our preliminary protein chemical data using differential labeling methods (Borst *et al.*, 1983a). Recently, we have found that the N-terminal amino acid sequencing and tryptic peptide maps of T3- δ and T3- ϵ are completely different (Borst *et al.*, 1984).

Both T3- δ and T3- ϵ appear to be T cell-specific structures. Obviously, a more extensive tissue screening needs to be conducted to confirm this. But since these polypeptides are thought to be part of a receptor complex that functions in T cells only, it is plausible that all components are indeed exclusively expressed on T lymphocytes. SP-64, like OKT3 and anti-Leu 4, intensely stained the medullary thymocytes and

cells in peripheral lymphoid tissue, and faintly stained most of the cortical thymocytes. Interestingly, WT-31 which detects a common determinant on all human T-cell receptors, showed similar staining of thymus. This supports the notion that the cell surface expression of T3 and T cell receptor is coordinated in the thymus. The SP-6 and SP-10 antibodies, which define T3- ϵ chain, showed staining patterns slightly different from SP-64 antibody. There appeared to be less staining for T3- ϵ than T3- δ on medullary thymocytes and T cells in peripheral lymphoid tissue. In contrast, the staining of cortical thymocytes for T3- ϵ appeared to be the same or possibly more than that for T3- δ . It cannot be deduced however, from staining of tissue sections whether thymocytes and peripheral T cells express T3 chains on their cell surface or whether the antibodies detect an intracellular protein. Furthermore, the intensity of staining obtained in tissues may not be directly related to the amounts of antigen present and may be affected by several factors including the fixatives used prior to staining and the affinity of the antibody.

The reagents that we have developed here are currently being used in studies of biosynthesis and function of the T3/T cell receptor complex. The monoclonal antibodies may be useful in attempts to isolate cDNA clones coding for the T3- ϵ chains. Better definition of the assembly of the functional T3/T cell receptor complex might provide insight into the mechanism of transmission of activation signals during the process of antigen-specific T cell recognition. Knowledge of the regulation of gene expression, and biosynthesis of the T cell receptor subunits will aid our understanding of T lymphocyte differentiation and leukemogenesis.

Materials and methods

Cells

Mouse myeloma cell line X63-Ag8.653 (Kearney *et al.*, 1979) and human leukemic cell line HPB-ALL (Morikawa *et al.*, 1978) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in a humidified 5% CO atmosphere at 37°C. Normal peripheral blood was obtained from healthy donors. Thymus, tonsils and lymph nodes were obtained from surgical procedures performed for diagnostic or therapeutic purposes. Lymph nodes and tonsils showed follicular and diffuse hyperplasia. Mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation ($d = 1.078$) (Boyum, 1968).

Purification of the T3 proteins

Large-scale preparation of the T3 proteins from (30×10^9) HPB-ALL cells was done as described (Snow *et al.*, 1984). HPB-ALL cells were obtained from the MIT cell culture facility and plasma membranes were prepared as described (Terhorst *et al.*, 1981). Purified plasma membranes were solubilized in 2% Nonidet-P40 (NP-40) and the soluble material was passed over 10 ml mouse IgG-Sepharose columns. The flow-through material was then absorbed on a 6 ml anti-Leu-4-Sepharose column, which was washed under several conditions (Terhorst *et al.*, 1981) and the T3 proteins were eluted with a 3.5 M MgCl₂, 0.2% NP-40 buffer. The detergent was removed by precipitation with ice-cold 10% trichloroacetic acid and repeated washes with acetone (Borst *et al.*, 1983a). After lyophilization this preparation, which contained mainly the 20-kD T3 proteins, was used for dot-blot experiments and some of the Western blots. The 20-kD T3 proteins were further purified by preparative SDS-PAGE and electroelution as described (Snow *et al.*, 1984). The latter material was used for immunization and Western blotting after Endo-F treatment.

Monoclonal antibodies

A 6-week old BALB/c female mouse was immunized with affinity-purified T3 by an i.p. injection of 10 μ g of protein in complete Freund's adjuvant. Booster injections were given at 3 week intervals in incomplete Freund's adjuvant. Three days after the last booster, spleen cells were fused with the non-secreting myeloma cell line X63-Ag8.653 (Kearney *et al.*, 1979) according to Kohler and Milstein (1975). Supernatants from the hybridomas were tested in a radioimmunoassay against crude T3 preparations. Selected hybridoma clones were subcloned three times by limiting dilution and banded

into mass cultures. Ascites ranging in titer from $1:10^4$ – $1:10^6$ were produced by injecting 2×10^8 hybridoma cells i.p. into heptane-primed BALB/c mice. The immunoglobulin isotype was determined by immunodiffusion in Ouchterlony plates using class- and subclass-specific goat anti-mouse immunoglobulin antisera (Meloy Laboratories, Springfield, VA).

Rabbit antibodies

New Zealand white rabbits were immunized with a synthetic peptide representing the 14 N-terminal amino acids of the chain coupled to KLH. Primary injection was given in the presence of complete Freund's adjuvant and boosters were given at 2 week intervals in incomplete adjuvant.

Dot-immunoblotting assay

CNBr-activated paper was prepared according to Anderson *et al.* (1979). A grid of squares (2×2 mm) was drawn on paper filters and ~ 10 ng of SDS-denatured T3 in 1 μ l volume was bound to each square with an overnight incubation in humid chamber at room temperature. All following steps were carried out at room temperature on a rocking platform. In order to reduce non-specific binding, filters were then incubated for 2 h in 'blocking buffer' (0.1 M Tris-HCl pH 7.6, 0.25% gelatin, 0.5% NP-40, 0.02% NaN₃). Each paper square was then cut and placed into one well of a 24-well tray and 100 μ l of hybridoma supernatants were added to each well and incubated for 2 h. After three washes, 30 000 c.p.m. of [¹²⁵I]sheep F(ab') anti-mouse Ig (Amersham) were added to each well and incubated for 2 h. After three washes, paper squares were dried and examined by autoradiography.

Peripheral blood lymphocytes, thymocytes and/or HPB-ALL cells were reacted with 20 μ l of the specific antibody and 20 μ l of fluorescein isothiocyanate (FITC)-conjugated goat F(ab') anti-mouse IgG (Cappel) as described (Spits *et al.*, 1983). The percentage of fluorescent cells was determined using either a Leitz fluorescent microscope or an Epics V cell sorter (Coulter Electronics, Hialeah, FL).

[³H]Thymidine uptake of peripheral blood lymphocytes in the presence of the monoclonal antibodies was described previously (Spits *et al.*, 1983).

Immunoperoxidase studies were carried out on acetone-fixed frozen tissue sections using avidin-biotinylated peroxidase complex method (Vector Labs., Inc., Burlingame, CA) as previously described (Cerf-Bensussan *et al.*, 1983).

Radiolabeling of cells

Cell surface labeling with [¹²⁵I]Na (ICN Radiochemicals, Irvine, CA) was catalyzed by lactoperoxidase as described previously (Borst *et al.*, 1982). For biosynthetic labeling, cells were washed and incubated for 1 h in methionine-cysteine-free medium and cultured for 8–16 h in the presence of 20 μ Ci/ml of [³⁵S]methionine and 20 μ Ci/ml of [³⁵S]cysteine.

Immunoprecipitation

Immunoprecipitation techniques were previously described in detail (Oettgen *et al.*, 1984).

Digestion with endoglycosidase F

T3 purified protein was resuspended in 0.1 M NaPO₄, 50 mM EDTA, 1% 2-mercaptoethanol, 0.1% SDS, pH 6.1 and boiled for 2 min. NP-40 was then added to 1%, and Endo-F digestion was carried out for 2 h at 37°C in the presence of protease inhibitors as described by Borst *et al.* (1983a).

Electrophoresis and autoradiography

Immunoprecipitates were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5–15% linear gradient slab gels according to a modification of the Laemmli procedure (1970). Two-dimensional electrophoresis was performed according to O'Farrell (1975). An Ampholytes mixture with pI ranges of 3.5–10, 4–6 and 5–8 was used in a ratio of 10:1:1. Samples were taken up in 8.8 M urea, 0.4% SDS, 5% 2-mercaptoethanol, 0.2% Ampholytes. After a 45 min incubation at room temperature, an equal volume of 8.8 M urea, 2% NP-40, 5% 2-mercaptoethanol and 2% Ampholytes was added and electrophoresis was performed. Samples were run from cathode to anode. The second dimension consisted of a 12.5% SDS-polyacrylamide gel. Gels containing proteins labeled with ³⁵S were treated with Amplify (Amersham) before autoradiography. Autoradiography was carried out at -70°C against Kodak XAR-5 film. Intensifier screens (Cronex Lightning Plus, DuPont Chemical Co., Newton, CT) were used for ¹²⁵I-labeled material.

Western blotting

After SDS-PAGE in a 'minigel' apparatus, proteins were blotted on nitrocellulose membrane at 60 V for 3 h in a Tris-glycine buffer (Towbin *et al.*, 1979). Filters were then saturated overnight with 3% bovine serum albumin and cut into small strips. Strips were incubated with antibodies for 2 h, washed four times, incubated with 2×10^6 c.p.m./strip of [¹²⁵I]protein A or [¹²⁵I]sheep anti-mouse IgG (Amersham) for 2 h, washed four times and subjected to autoradiography. The procedure was carried out at room temperature on a rocking platform. For sequential Western blotting, an-

tibodies were removed from the filters with a 20 min wash in 20 mM triethylamine buffer pH 11.5.

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