Immunohistological detection of human cytotoxic/suppressor T cells using antibodies to a CD8 peptide sequence

D Y Mason, J L Cordell, P Gaulard, A G D Tse, M H Brown

Abstract

Aims: To evaluate whether cytotoxic/suppressor T cells can be detected in paraffin wax embedded human tissue samples using antibodies to a synthetic CD8 peptide sequence.

Methods: Polyclonal and monoclonal antibodies were raised against a 13 amino acid peptide sequence from the cytoplasmic portion of the α chain of the human CD8 molecule.

Results: These antibodies specifically detected the native form of the CD8 polypeptide when tested by immunoprecipitation with radiolabelled T cells, and gave the expected staining pattern for cytotoxic/suppressor T cells in cryostat sections. Being raised in rabbits, the polyclonal antibodies were also useful for double labelling for CD8 in conjunction with monoclonal antibodies. CD8 positive cells could also be detected in paraffin wax embedded tissues. This was achieved without prior treatment of the sections if the tissue had been fixed in Bouin's fixative. When tissues had been exposed to conventional formalin fixation, preliminary microwave treatment was required.

Conclusions: These findings provide further evidence that antibodies against leucocyte associated antigens, capable of reacting on paraffin wax embedded tissue, can be produced by immunisation with synthetic peptide sequences.

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Most monoclonal antibodies against human white cell associated antigens used for the immunohistological study of lymphoproliferative disorders are not suitable for use on routinely processed paraffin wax embedded tissue. This is because the epitopes which they recognise are sensitive to denaturation. We have reported how this obstacle can be overcome in the case of the pan T cell marker CD3 by the preparation of polyclonal rabbit antibodies against a peptide sequence from this molecule.¹ These antibodies labelled T cells strongly in routinely fixed paraffin wax embedded tissue and proved of value in the diagnosis of human T cell lymphomas. The sequence selected for immunisation was from a cytoplasmic region of the CD3 molecule and this may account for the resistance of the epitope to denaturation, in that such sequences are not glycosylated and are also likely to be in an

extended conformation, mimicked by a linear peptide. In contrast, extracellular regions of protein, against which most monoclonal anti-leucocyte antibodies are directed, tend to be tight folded, or glycosylated, or both.

We have now explored this further by preparing antibodies against a 13 amino acid peptide sequence from the C terminal region of the α chain of another leucocyte associated molecule, the CD8 antigen, a marker of cytotoxic/suppressor T lymphocytes. We have produced both polyclonal and monoclonal antibodies which react strongly and selectively with CD8 when tested in immunoprecipitation experiments and by immunohistological staining of tissue sections. These antibodies can also be made to label cytotoxic/suppressor T cells in routinely processed paraffin wax embedded tissue.

The polyclonal antibodies against CD8 were also of value for rapid double immunoenzymatic staining of tissue sections because they were of rabbit origin and could therefore be used in combination with monoclonal antibodies to other white cell markers without the problems inherent in using two primary antibodies raised in the same species.

Methods

PEPTIDE PRODUCTION

The peptide corresponding to the 13 C-terminal amino acids of the cytoplasmic domain of the CD8 chain was synthesised on an Applied Biosystems peptide synthesizer (Foster City, California) model 430A. Its sequence was as follows: Cys-Lys-Ser-Gly-Asp-Lys-Pro-Ser-Leu-Ser-Ala-Arg-Tyr-Val.² The terminal cysteine was added to allow conjugation to carrier proteins. The synthetic peptide was purified by reverse phase high pressure liquid chromatography before use. The peptide was coupled to thyroglobulin and to bovine serum albumin using m-maleimidobenzoyl-N-hydroxysulfo-succinimide ester [sulfo-MBS] and sulfo-succinimidyl-4-(N-maleimodomethyl) cyclohexane-1-carboxylate [sulfo-SMCC], respectively. The amount of peptide coupled to the protein carriers was determined by amino acid analysis and expressed as percentage weight of peptide to the weight of the final peptide conjugates. The values for the thyroglobulin and BSA conjugates were 28% and 36%, respectively.

IMMUNISATION

Two rabbits were immunised on three occasions at intervals of two weeks by subcutaneous

LRF
Immunodiagnostics
Unit, Department of
Haematology, John
Radcliffe Hospital,
Oxford, OX3 9DU
D Y Mason
J L Cordell

Department of
Pathology, CHU Henri
Mondor, Creteil,
France
P Gaulard

MRC Cellular
Immunology Unit, Sir
William Dunn School
of Pathology,
University of Oxford
A G D Tse
M H Brown

Correspondence to:
Dr D Y Mason

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injections of 200 µg of the thyroglobulin-peptide conjugate emulsified in Freund's complete adjuvant. Serum was obtained two weeks after the third immunisation. Balb/c mice were immunised on three occasions at 10 day intervals with 50 µg of thyroglobulin-peptide conjugate emulsified in Freund's complete adjuvant followed by a final intraperitoneal booster dose (100 µg). A mouse was then sacrificed, its spleen removed, and cell fusion performed with the NS1 myeloma cell line by a conventional technique, as described previously.³ Culture supernatant fluids were screened on cryostat sections of human spleen by immunohistological staining using the alkaline phosphatase-antialkaline phosphatase (APAAP) technique.^{4,5}

AFFINITY PURIFICATION OF POLYCLONAL ANTI-PEPTIDE ANTIBODY

The CD8 peptide-BSA conjugate was coupled to CNBr-activated Sepharose (Pharmacia) at 5 mg/ml Sepharose. The gel was blocked with 100 mM ethanolamine HCl (pH 8.5) and washed in TRIS-buffered saline (TBS), pH 7.4. Rabbit serum (1.2 ml) was passed down a 0.5 ml CD8 peptide-BSA-Sepharose column and the column washed with TBS (pH 7.4). Bound material was eluted with 4M MgCl₂. The MgCl₂ was then replaced with TBS by gel filtration, giving a final yield of 3.5 mg of protein.

TISSUE SAMPLES

Cryostat and routinely processed paraffin wax embedded sections of formalin fixed or Bouin's fixed tissue were obtained from the histopathology departments of the authors' hospitals.

IMMUNOHISTOLOGICAL STAINING

Staining was performed by the APAAP procedure.^{4,5} In some experiments paraffin wax sections were incubated with trypsin or with pronase for between 10 and 30 minutes before staining.⁶ Heating of sections by exposure to microwaves was also used for some samples before immunostaining.⁷ CD8 peptide antisera were used at a range of dilutions between 1 in 100 and 1 in 400 and affinity purified antibodies were used at a concentration of 12 µg/ml. Incubation with antisera or affinity purified antibodies was carried out at room temperature for either 30–60 minutes or overnight. Sections stained with rabbit anti-CD8 were incubated with mouse monoclonal antibody against rabbit Ig, and sections were then incubated with the reagents for the APAAP technique.

Double immunohistological labelling was performed by incubating cryostat tissue sections with a mixture of polyclonal anti-CD8 antibody and monoclonal antibody (either anti-CD3 or anti-CD4). Sections were then washed and incubated with a mixture of peroxidase conjugated swine anti-rabbit Ig (Dakopatts) and alkaline phosphatase conjugated goat anti-mouse Ig (kindly provided by Dr K-J Pluzek), both diluted 1 in 20. Sections were then washed and incubated in turn with

substrates for the peroxidase and alkaline phosphatase reactions.

IMMUNOPRECIPITATION OF CD8 ANTIGEN

Human T lymphoblasts were generated from peripheral blood mononuclear cells using phytohaemagglutinin (PHA) and subjected to radioiodination and immunoprecipitation, as described previously.⁸ Each precipitate was prepared from 10⁷ cells labelled by lactoperoxidase catalysed iodination with 400 µCi¹²⁵I (IMS 30 Amersham International UK) and lysed in 1% Nonidet P40 or with 0.5% digitonin. Precipitates were washed under stringent conditions (10 mM TRIS-HCl(pH 7.5) containing 0.5 M NaCl, followed by 10mM TRIS-HCl(pH 7.5), 0.15 M NaCl containing 0.1% sodium dodecyl sulphate (SDS)) and analysed on a 12.5% sodium dodecyl sulphate polyacrylamide gel under reducing conditions. Bands were detected by autoradiography.

Results

Affinity purified polyclonal rabbit antibodies to the CD8 peptide were prepared as described in Methods. Hybridoma cultures from the spleen cells of a mouse immunised with the peptide conjugate were screened on spleen and tonsil tissue sections. Three positive cultures were obtained which yielded three stable cell lines (designated C8/123G, C8/123J, and C8/144B on cloning).

Both polyclonal and monoclonal antibodies to the CD8 peptide precipitated material from radiolabelled T lymphocytes which migrated in SDS gels with a molecular weight of about 32 kilodaltons (fig 1).

The affinity purified rabbit antibodies and the mouse monoclonal antibodies produced immunohistological staining reactions on cryostat sections of human spleen and tonsil which were identical in pattern and intensity with those produced with conventional CD8 monoclonal antibodies—that is, labelling of scattered cells in T cell areas and of splenic sinusoidal lining cells (fig 2). The only exception was that two of the monoclonal antibodies (C8/123G and C8/123J) also both stained basal epithelium in tonsil. The polyclonal antibodies also stained Bouin's fixed, paraffin wax embedded tissue (figs 3–5D), although there was some variation in the strength of labelling among different biopsy specimens, occasional samples failing to give satisfactory staining. Proteolytic digestion did not enhance reactivity in Bouin's fixed sections. Formalin fixed, paraffin wax embedded tissue sections did not stain with the polyclonal or monoclonal anti-CD8 antibodies, whether or not they were subjected to preliminary proteolytic digestion. However, CD8 positive cells could be labelled in this type of tissue when sections had been heated in a microwave oven before immunostaining (fig 5E).

Double staining of T helper and suppressor cells using a mixture of the polyclonal anti-CD8 antibody in combination with mono-

Figure 1 ¹²⁵I immunoprecipitates from human T lymphocytes prepared with monoclonal (reagent C8 123G) and polyclonal anti-CD8 peptide antibodies (tracks A and B, respectively) and with the anti-CD3 antibody OKT3 (track C). The 32 kilodalton band immunoprecipitated in tracks A and B is of the appropriate size for the CD8 α chain coprecipitating with CD8- β chain.

clonal anti-CD3 or anti-CD4 in a two step immunoenzymatic method is illustrated in fig 6.

Discussion

The results indicate that antibodies produced by immunisation with a synthetic peptide are virtually indistinguishable, in terms of their immunocytochemical reactivity on tissue sections and their biochemical reactions, from those of conventional CD8 monoclonal antibodies. The principal aim of this study was to investigate whether antibodies to the CD8 C-terminal peptide would be suitable for use on routinely processed human tissue. We found that the polyclonal antibodies gave good labelling of Bouin's fixed, paraffin wax embedded

tissue, and initially concluded that formalin fixation destroyed or irreversibly masked the epitope recognised by the CD8 antibody. However, experiments performed near the conclusion of this study showed that prior heating of sections in a microwave oven offered a simple means of rendering the epitope detectable (fig 5E).

Polyclonal antibodies to a synthetic peptide sequence which recognise mouse CD8 by immunoprecipitation and western blotting have been reported by Brunati *et al*, but no data were given on the detectability of this molecule in fixed paraffin wax embedded tissue.⁹ Ward *et al* have described the detection of CD8 positive cells in routinely fixed paraffin wax embedded tissues using the monoclonal antibody OX-8,¹⁰ but this was performed on rat tissues and we are not aware of any previous reports of CD8 detection in paraffin wax embedded human tissues.

As the polyclonal CD8 antibodies were raised in rabbits, they were useful in double labelling experiments. Normally, when pairs of monoclonal antibodies are used for such experiments, problems arise due to the difficulty in distinguishing between the two primary reagents. This problem may be overcome if the two monoclonal antibodies are of different isotype or subtype (for example IgM and IgG, or IgG1 and IgG2a), although good labelling requires specific secondary antibodies. Double staining can also be carried out sequentially—by performing an immunoperoxidase reaction first with one monoclonal antibody and then an immunoalkaline phosphatase sandwich with the other, but this approach is time consuming. A third approach, which is only suitable when substantial amounts of monoclonal antibody are available, involves conjugation of the two monoclonal antibodies with biotin or a hapten (arsanilate) and their detection with reagents such as avidin and anti-hapten antibodies.

Consequently, the availability of a rabbit antibody against CD8 greatly facilitates double labelling because high quality anti-rabbit second stage reagents labelled with different markers (both fluorescent and enzymatic) are

Figure 2 Immunohistological labelling of human spleen (infiltrated by B cell lymphoma) with polyclonal anti-CD8 peptide antibody. Scattered lymphoid cells and also sinusoidal lining cells are stained (cryostat section, APAAP method).

Figure 3 Small bowel tissue stained with polyclonal anti-CD8, showing staining of intraepithelial lymphocytes, (paraffin wax section of Bouin's fixed tissue, APAAP method).

Figure 4 CD8 positive T cell lymphoma stained with polyclonal anti-CD8 (paraffin wax section of Bouin's fixed tissue, APAAP method).

Figure 5 (A–D): Paraffin wax embedded, Bouin's fixed lymph node tissue showing adjacent sections stained for CD3 (A and C) and CD8 (B and D), using polyclonal anti-peptide antibodies (APAAP method). (A) and (B): part of a lymphoid follicle and an adjacent interfollicular area are seen. G = germinal centre. (C) and (D): T cell area. High endothelial venules are marked with arrows.

(E): Left: paraffin wax embedded, formalin fixed tonsil stained with monoclonal anti-CD8 showing scattered positive cells in the T cell area. G = Germinal centre. Right: negative control staining on an adjacent section (Immunoperoxidase staining following prior heating in a microwave oven).

available from a variety of sources and can be used in conjunction with a conventional immunocytochemical method for the monoclonal antibody. In this study, we were able to mix the primary antibodies and the enzyme conjugated antibodies by which they were detected, thus enabling double labelling to be carried out as a four stage technique (two antibody stages followed by two enzyme substrate reactions).

The antibodies reported in this paper have one other advantage, compared with other CD8 reagents, in that they react with a defined portion of the molecule (the terminal 13 amino acids of the cytoplasmic tail). They could therefore be used to study the interaction of this portion of the CD8 molecule with other leucocyte associated molecules. In this context it may be noted that evidence has been reported^{11 12} for interaction between CD8 and the protein-tyrosine kinase p56^{lck}, and the antibodies described in this paper could be used to explore this in more detail.

Figure 6 Double immunoenzymatic staining of T helper and T suppressor cells in human tonsil (cryostat section) using polyclonal anti-CD8 (brown reaction product) in combination with either monoclonal anti-CD4 (A) or monoclonal anti-CD3 (B) (red reaction product). T helper cells in the germinal centre in (A) are mixed with CD4 positive macrophages (arrows). This problem is avoided in (B) when helper cells are identified as CD3⁺ CD8⁻ cells, as germinal centre macrophages (arrow) are unstained. (C) A T cell area from the same section as (B) stained for CD3 and CD8 clearly showing the two cell populations.

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