CHAPTER 7

THEORETICAL BACKGROUND AND EXPERIMENTAL EVALUATION OF THE TECHNIQUE FOR ULTRASTRUCTURAL IMMUNOLOCALISATION OF THE H-SUBUNIT AND L-SUBUNIT OF FERRITIN

1) Introduction

Ferritin, the major intracellular protein responsible for the storage of iron in all cell types consists of 24 subunits of various combinations of two types, the H-subunit and the L-subunit (Chapter 1). The combination of these two subunits in the ferritin protein shell plays an important role in ferritin's iron handling capabilities. In the bone marrow, iron is differently metabolised by the cells of the erythron including erythroblasts, reticulocytes and red blood cells on the one hand, and the macrophage on the other. Furthermore, iron is shuttled between the cells of the erythron and the macrophage to support erythropoiesis. In order to investigate the role of the H-subunit and the L-subunit of ferritin in the handling of iron by cells of the bone marrow it is necessary to investigate the expression of the two subunits of ferritin at the single cell level.

Ultrastructural electron microscopy can be employed to distinguish the different cells of the bone marrow from one another and, more importantly, to localise signals generated

from the different subunits to a specific cell. In this study, ultrastructural immunolocalisation was employed to investigate the expression of the H-subunit and L-subunit of ferritin in different cell types in the bone marrow.

2) Theoretical background of the technique for the immunolocalisation of the H-subunit and L-subunit of ferritin

The technique for the immunolocalisation of the H-subunit and L-subunit of ferritin had to be developed, since this technique was not available at the University of Pretoria. A thorough background study was undertaken to assist in the development and evaluation of this technique. This chapter presents an overview of the relevant background followed by the experimental evaluation.

2.1) Preservation of bone marrow tissue and protein for immunolocalisation

Ultrastructural immunolocalisation depends on well-preserved structural detail together with intact and recognisable antigens for proper binding of the antibody. The preservation of structural detail of tissue for transmission electron microscopy involves different steps each with its own purpose. These steps include fixation, dehydration and embedding. The aim of the fixation step is to stabilise cellular organisation to such an extent that ultrastructural relations are preserved despite the subsequent rather drastic treatments of dehydration, embedding and exposure to the electron beam (1). During the process of fixation the various side-groups of proteins are linked together, either intra-molecularly or inter-molecularly, to form a meshwork of proteins, other cellular molecules and organelles. After fixation the tissue is dehydrated, since most electron microscopy resins are not miscible in water. Dehydration of the fixed tissue will result in the removal of all water molecules whereupon the tissue is infiltrated with the resin. Once complete infiltration of the resin has occurred the resin is polymerised. The tissue

is embedded in a resin suitable for cutting thin sections of no more than a 100 nm for viewing by an electron microscope. All these procedures necessary for the preparation of the tissue for ultrastructural microscopy can have deleterious effects on the antigen resulting in the loss of antibody binding. Therefore, in order to successfully immunolocalise an antigen, a compromise has to be reached between the preservation of ultrastructural detail and the retaining of antigenicity.

2.2) Ultrastructural immunolocalisation of antigens

Various factors should be taken into consideration with the immunolocalisation of an antigen. These factors include an intact antigen at the end of the preparation procedure with minimal change in antigenicity, negligible translocation of the antigen and the accessibility of the antigen to the antibody. Furthermore, should the ultrastructural detail be preserved as close to the natural situation as possible (2). For ultrastructural immunolocalisation of antigens three well characterised methods have been developed. However, each of these procedures has their own advantages and limitations. These procedures include pre-embedding immunolabelling, post-embedding immunolabelling and cryo-immunolabelling. With pre-embedding immunolabelling the tissue is fixed followed by the immunolabelling procedure before dehydration, embedding and sectioning. Limiting factors include low antibody penetration necessitating the need for permeabilising procedures with detergents. This can result in irregular labelling, since the permeabilisation step does not always bring about homogenous antibody penetration of the tissue. The permeabilisation step can also result in loss of ultrastructural detail. The advantage of pre-embedding immunolabelling is that the antigen has not been exposed to the organic solvents used in dehydration and embedding procedures before immunolabelling takes place. For post-embedding immunolabelling the immunolabelling takes place only after fixation, dehydration, embedding and sectioning. The limiting

factor with post-embedding immunolabelling is that significant changes can be caused to the antigen's structure that can result in a decrease in binding of the antibody to the antigen. The advantage of post-embedding immunolabelling is that no tissue permeabilisation step is necessary to bring about the penetration of the antibody into the tissue. The third of these methods, cryo-immunolabelling, is considered to be the method giving superior results when compared to the first two methods. Firstly, the antigen is preserved the best during cryo-treatment of the tissue since the antigen is not exposed to any of the harsh treatments before the immunolabelling step takes place. Secondly, the cryo-sections are fully penetrable to antibodies. Nevertheless, a disadvantage with cryo-immunolabelling is that the antigen may be extracted during the immunolabelling step as a result of the weak fixation and absence of embedding (3, 4).

2.3) Steps in preservation of the tissue and antigen for post-embedding immunolabelling

For the present study post-embedding immunolabelling was chosen, since pre-embedding immunolabelling is more disadvantageous for quantifying the immunolabelling due to possible in-homogenous antibody penetration and the facilities for cryo-immonolabelling were not available at the time of the study. However, any manipulation of the tissue during its processing for post-embedding immunolabelling including the fixation, dehydration and embedding procedures can result in modifications of tissue components. Proteins are extremely prone to these modifications and as a consequence the binding of antibody to antigens can be changed or even completely lost. Furthermore, these procedures can affect different antigens to different extents. Therefore, conditions for optimal immunolabelling must be evaluated, which usually is a compromise between ultrastructural preservation and retention of antigenicity (3, 5). However, there are a number of theoretical guidelines.

2.3.1) Fixation

One of the purposes of fixation is to secure the antigen where it naturally occurs. However, one of the consequences of vigorous fixation may be that the antigen is damaged or altered so that it is unrecognisable or inaccessible to antibodies (6). For optimal preservation of ultrastructure the method of choice is generally primary fixation in a glutaraldehyde (GA) solution of more than one percent followed by post-fixation with osmium tetroxide (5). However, both of these substances can have a deleterious effect on immunolabelling efficiency. Glutaraldehyde, a di-aldehyde containing two free aldehyde groups, can bind to cellular components with both of these aldehyde groups. It is by binding to cellular components, such as amino acid side-chains, with both of these aldehyde groups, that extreme cross-linking occurs bringing about good preservation of the ultrastructure, but possible loss of immunolabelling efficiency. Nevertheless, not all antigens are affected by GA, but some antigens are extremely sensitive to cross-linking by GA so that the concentration of GA has to be reduced or, in the most extreme cases, omitted entirely. A fixative containing 0.5% GA is generally suitable for a wide variety of antigens (7). It has been shown that even lower concentrations of GA (< 0.2%) allow the labelling of not only the primary or high concentration antigenic sites, but also the secondary or low concentration antigenic sites, while higher concentrations of GA (> 1%) only allow labelling of the primary sites (8). When these low concentrations of GA are used, formaldehyde (FA) is added. Formalin is not added since it contains methanol, another substance deleterious to antigens (7). FA alone preserves the ultrastructure poorly, which is most probably due to the reversibility of the majority of formaldehyde-induced cross-links (2). For optimal ultrastructure preservation this primary fixation step is followed by fixation in osmium tetroxide. Osmium tetroxide fixes cellular lipids, especially those forming part of the cell membrane. All of these lipid-rich structures are poorly preserved by GA and FA therefore osmium post-fixation compensates for this

disadvantage. However, the use of osmium tetroxide is not advised for immunolabelling techniques.

In addition to composition, exposure time and temperature of fixation can also affect the ability to immunolabel a particular antigen. It is therefore preferable to fix tissues by perfusion, but if that is not possible, fixation at cold temperatures seem to reduce tissue degradation before proper stabilisation of the tissue has occurred. By keeping the time between removal of the tissue and fixation as short as possible, further tissue decomposition can be prevented before proper fixation (7).

2.3.2) Dehydration

Dehydration by an organic solvent follows the fixation of the tissue. This is necessary since the embedding solutions are not miscible in water. The tissue is therefore dehydrated by an appropriate organic solvent before the embedding procedure. The extent of this dehydration process is important when proteins are minimally cross-linked for the subsequent immunolabelling procedures. The advantages of minimally cross-linked proteins for subsequent immunolabelling procedures are lost if room temperature, complete dehydration protocols are used. Room temperature, complete dehydration can result in extraction of the minimally cross-linked antigens. This can be prevented if either, room temperature, partial dehydration or progressively lower temperature (PLT) protocols are followed. Both these methods are probably able to reduce extraction of soluble tissue components. In addition partial dehydration could also be performed at cold temperatures. This would result in further reducing the extraction of minimally cross-linked material. Lipid retention is said to be higher with partial dehydration. It has been shown that the worst effects of ethanol dehydration occur (shrinkage and other dimensional changes) when it is in excess of 70% and that it is important to avoid the

extreme concentrations of ethanol in order to best preserve the reactivity of the tissues to antibodies (9). Disruption of protein conformation is minimised by performing dehydration at low temperatures. At the low temperatures used for PLT protocols the hydration shells around proteins may be better preserved in an organic solvent than in the corresponding solvent concentration at room temperature. The fall in the dielectric constant at progressively lower temperatures is not as marked as the corresponding fall at room temperature with the use of 100% organic solvents (8).

2.3.3) Embedding

The choice of embedding agent is important for optimal immunolabelling. For post-embedding immunolabelling procedures the hydrophilic resins such as LR White, LR Gold and Lowicryl generally give better results than the epoxy-based hydrophobic resins such as the Epon substitutes or Spurr (6, 7, 10). There are a number of factors responsible for the attainment of better immunolabelling with the hydrophilic resins. LR White is compatible with about 12% by volume of water so that partial dehydration with an organic solvent is possible. The ability of some resins to tolerate water to a lesser or greater extent may create conditions for complementing a region of polarity (or water) around molecular structures. It is these properties of the acrylic resins that could be responsible for allowing a degree of hydration to be retained within the tissues, thus increasing the chances for improvements in ultrastructure and immunoreactivity (8). Furthermore, LR White is less lipophilic than epoxides and therefore less likely to disrupt ultrastructure by extraction of especially lipids when post-fixation with osmium tetroxide is omitted (2, 6). In addition LR White does not form covalent bonds with biological material during the polymerisation procedure. This is in contrast with epoxies that form covalent bonds with biological material. The epoxies particularly form covalent bonds with proteins, resulting in co-polymerisation instead of a polymerised mixture. The

reactive epoxy groups have a great tendency to react with hydroxyl and amino groups, which are chemical side-groups present in biological macromolecules such as proteins and nucleic acids. Therefore, the bio-molecules will be part of the polymer network when embedded in epoxy resin. LR White polymerises by free radical chain polymerisation, these free radicals react with double bonds of the acrylic monomer and have no affinity for proteins and nucleic acids. Thus, bio-molecules are not incorporated into the polymer network (11).

2.3.4) Curing of the resin (polymer cross-linking)

In the present study the embedding medium of choice was LR White. Different procedures are possible for curing of LR White. Firstly, LR White can be heat cured at 50°C with complete polymerisation within 24 hours. Secondly, due to the presence of benzoyl peroxide in the LR White solution, LR White can be chemically polymerised by the addition of an accelerator. When accelerator is added to pre-cooled LR White monomer (-20°C) the mixture begins to gel after about 30 minutes and the polymerisation procedure is complete after 24 hours at -20°C (LR White remains liquid to about -20°C). However, the end of the capsules distal to the tissue always retains a small amount of un-polymerised resin. This catalytic reaction is exothermic. Therefore, to ensure dissipation of heat the capsule should be placed in a cold aluminium block with drilled holes (8). Thirdly, polymerisation of LR White is possible with exposure to UV-light. Performance of this procedure can be accomplished at different temperatures and, depending on the temperature, the time of polymerisation of the resin can be manipulated. At -20°C polymerisation is completed in seven days whereas at 4°C and 20°C polymerisation is completed in four days and eight hours, respectively (12). For all three these curing procedures there exists different advantages and drawbacks. For both

the heat and UV-light curing procedures a decrease in immunolabelling efficiency is shown for certain antigens, which may be explained by a damaging influence of the monomer on antigenic sites. It is thought that prolonged exposure to the plastic monomer may cause considerable extraction of cellular material from the tissue (9, 12). This is not the case for the rapid chemical curing procedure. However, care must be taken because this is an exothermic reaction and will give of heat – sometimes damaging antigenicity. Furthermore, the use of excessive accelerator can result in over cross-linking (9). However, it has been shown that once the tissue is fixed and dehydrated, high temperatures do not damage the tissue any more. Significant denaturation is shown to occur only at temperatures above 70°C in unfixed purified proteins compared to virtually no effect on formalin-fixed proteins (13). For the low temperature curing procedures improved preservation of fine structural details of mildly aldehyde-fixed tissues is shown, together with superior preservation of sensitive antigens (14, 15). The degree of LR White cross-linking attained with curing can be changed by the polymerisation conditions (9). In theory the lower the cross-link density of the resin, the more penetrable it will be to aqueous solutions and therefore to immunoreagents. For example, slow curing at 50°C for 24 hours prevents LR White from becoming completely cross-linked and also induces linearity in its molecular arrangement, which favours penetration by aqueous solutions (9). Due to different methods of polymerisation the extent of polymer cross-linking can be changed so as to increase the sensitivity and ease of post-embedding immunolabelling (6). Hydrophilic plastics swell in aqueous solutions and the extent to which they swell depends on the extent to which they are cross-linked. Swelling may be the means by which immunoreagents gain access to antigens in the interior of LR White sections. Re-sectioning immunolabelled sections of rapidly embedded tissue has shown that the freely diffusible reagents of the immunoperoxidase method do penetrate into LR White. This could be the reason for

the greater sensitivity of this method compared to methods making use of colloidal gold and could also account for the high electron density that the diamino benzidine (DAB) deposited in ultra-thin sections can achieve (6). Therefore, no difference in immunolabelling efficiency is seen for different curing procedures with immunogold labelling since the gold particle is too large to penetrate the sections – no matter the extent of cross-linking achieved during the curing of the resin. However, with immunoperoxidase immunolabelling, slow-heat polymerisation will generally produce greater sensitivity to antibodies (9).

2.4) Surface relief upon sectioning and exposure of the antigen

With sectioning of the embedded tissue the surface of cleavage tends to follow the areas of least resistance, e.g., the interfaces between the resin and proteins (10). This tends to release the antigens from the embedding media resulting in the antigen being available for binding to an antibody – the stronger the surface relief the better the immunolabelling. The amount of corrugation – the depth of relief – is determined by the strength of co-polymerisation between the resin and proteins. With the strength of co-polymerisation determined by the chemical nature of the resin, the temperature at which curing takes place and the characteristics of the biological material that can be influenced by the process of fixation (16). For epoxy resins, with a large amount of co-polymerisation, sectioning results in little corrugation and surface relief. This results in hiding of antigens by thin layers of resin. For LR White, sectioning can result in a surface relief of 2-6 nm, which could be as much as three times more than that for epoxies. With this increase in the surface relief depth, antigens are more exposed for recognition by an antibody (8). Curing at low temperatures possibly causes a decrease in co-polymerisation followed by an increase in the depth of corrugation and an increase in immunolabelling. With low temperatures it is possible that the hydration shells that

surround all biological macromolecules suspended in aqueous cytosol, is not completely removed. This persisting shell of organised "ice-like" water could produce a sort of insulation against co-polymerisation at low temperatures. During cleavage of the resin this interspace between the antigen and the cured resin will favour the exposure of antigenic sites (16). Furthermore, the characteristics of the biological material determine the magnitude of co-polymerisation. Changing these biological characteristics by altering the surface of an antigen through fixatives such as aldehydes could alter its reactivity towards the resin, most probably increasing the ability to co-polymerise (16).

2.5) Antibody penetration of sections

Relative rapid penetration of antibodies into LR White sections is claimed in some reports, while no or only little penetration was observed in other studies (10). Due to the hydrophilic nature of LR White, it is suggested that the penetration of aqueous solutions could occur into thin sections of LR White embedded tissue (2). An indication of this penetrability of LR White is the swelling that occurs upon exposure to aqueous solutions. Furthermore, the formation of diamino benzidine (DAB) reaction product arising from horseradish peroxidise oxidation has been shown to not only cover the surface of the sections, but also to form within the resin (17). This penetrability of LR White to aqueous solutions makes it possible for antibodies to gain access into the plastic sections (10). This is important, as it has been suggested that the immunolabelling efficiency depends on how well immunoreagents are able to penetrate the section (17). However, the question remains as to whether LR White sections allow the penetration of gold-conjugated antibodies to the interior of the section. By comparing immunogold immunolabelling with immunoperoxidase immunolabelling, the immunoperoxidase reaction continues to be positive with primary antibody dilutions well beyond the detection sensitivity of the immunogold immunolabelling system (9). This can be

explained by the fact that, due to the size of the gold particulate, the immunogold immunolabelling reaction is confined to the exposed surface of the section. The immunoperoxidase label is not bigger than the size of an antibody and therefore it would be expected to be able to penetrate the sections. This would result in the labelling of antigenic sites not only on the surface of the section but also in the interior of the section (9). Therefore, if immunocolloidal gold is used the immunoreaction is immediately confined to the surface, in which case little difference will be made to the intensity of labelling by altering the polymerisation schedule (3, 9, 18).

2.6) Post-embedding procedures for increasing antigen availability

The ideal is to employ the most appropriate fixation, dehydration and embedding procedure in order to ensure optimal immunolabelling of the antigen in question. However, there are a few procedures available for increasing antigen availability for immunolabelling procedures when the fixation of the tissue is not optimal or a hydrophobic embedding medium has been used.

2.6.1) Etching of epoxy sections and removal of osmium tetroxide

Although the use of epoxy as an embedding medium for post-embedding immunolabelling procedures is theoretically not desirable, various antigens have been successfully immunolabelled with epoxy sections. However, immunolabelling is frequently only achieved once the bonds between the tissue and embedding media are broken – which is accomplished by etching (partial corrosion) of the section (6, 19). Etching pre-treatments involves a procedure whereby the resin (plastic) is removed upon breaking of the ester bonds, thereby decreasing the cross-linking density and increasing the hydrophilicity of the section (2, 5). Increased hydrophilicity of the section surface increases the accessibility of the antibody to the antigen. Partial corrosion of the epoxy

resin of the section can be achieved by exposing the section for ten seconds to saturated sodium ethoxide diluted to 50% with absolute ethanol (4), or by oxidising agents such as sodium metaperiodate, hydrogen peroxide or periodic acid. Treatment with a saturated aqueous solution of sodium metaperiodate for 30 to 60 minutes or with 1% aqueous periodic acid for 4 minutes results in successful immunolabelling (15). Deplastisising of epoxy sections with sodium ethoxide is possible because of the sensitivity of the ester bonds in polymerised epoxy resin to strong alkaline solutions. However, the rough treatment with sodium ethoxide may be harmful to sensitive antigens (20). By moderately increasing the amount of accelerator used during polymerisation, the amount of co-polymerisation of epoxy with the tissue can be decreased, resulting in superior immunolabelling with less need for harsh etching procedures with immunolabelling of sensitive antigens (21). In addition to the treatment with sodium ethoxide, it has been shown that the intensity of immunolabelling of epoxy sections can also be enhanced by heating of the sections in citrate solution. It is suggested that the mechanism for increasing the immunolabelling intensity on epoxy sections by heating is similar to the other etching procedures, i.e., by breaking the chemical bonds between the epoxy resin and the antigens (22). The following etching procedures were compared for superiority of immunolabelling of 2% glutaraldehyde fixed, 1% osmium tetroxide post-fixed and epoxy resin embedded tissue. The etching procedures included sodium metaperiodate, microwave irradiation in citrate buffer pH 6, microwave irradiation in EDTA solution pH 8 and microwave irradiation in an alkaline solution pH 10. Only heating of the sections in the alkaline solution pH 10 was shown to result in improvement and adequately labelled sections (23). In order to accomplish maximal immunolabelling with etching it is necessary to determine the right balance between the concentration of the etching agent and the etching time. This in order to provide sufficient permeability of the surface of thin sections for antibody access while avoiding structural damage, and to

retrieve antigens hidden by covalent bonds formed between the epoxy resin and biological material during polymerisation (4).

Not only are the epoxies heavily cross-linked, very hydrophobic and of a high tendency to form cross-links with tissue antigens, they are also strongly lipophilic. Due to this strong lipophilic nature of the epoxies, the tissue is also almost always post-fixed with osmium tetroxide to prevent the extraction of lipid cellular structures (6). However, post-fixation with osmium tetroxide results in complete loss of antigenicity. Nevertheless, oxidation of the reduced osmium could result in the restoration of the antigenicity. Oxidising agents oxidise the reduced osmium, which then becomes soluble and is then rinsed from the section (5, 24). By comparing osmicated and un-osmicated tissues embedded in epoxy resin it is shown that in addition to etching with 1% sodium ethoxide the osmicated sections also had to be oxidised with sodium metaperiodate for 60 minutes plus 5 minutes on 3% hydrogen peroxide at room temperature (25). Bendayan and Zollinger compared the following oxidising agents/procedures for their ability to retrieve antigens from glutaraldehyde-fixed post-osmicated tissues a) incubation for 10, 30, 60 and 120 minutes on a saturated aqueous solution of sodium metaperiodate, b) incubation for 10 minutes on a 10% solution of hydrogen peroxide, c) incubation for 2 minutes on a saturated alcoholic solution of sodium hydroxide diluted 1:10 with 100% ethanol, followed by successive incubations of 2 minutes in 100%, 95%, 75% and 50% ethanol, rinsed in distilled water and finally incubated for 5 minutes on 10% hydrogen peroxide and d) incubation for 10 minutes on a 5% solution of periodic acid. Treatment with the saturated aqueous solution of sodium metaperiodate for 30, 60 or 120 minutes restored the labelling without altering the structural information, with the restoration of labelling being time-dependent: after 10 minutes of treatment, the intensity of labelling was about half of that obtained after 30 minutes and maximal labelling intensity was

reached after 60 minutes. The other strong oxidising agents tested including the hydrogen peroxide and periodic acid and the combination of alcoholic solution of sodium hydroxide followed by hydrogen peroxide were also found to restore the labelling. However, in contrast to the results obtained with sodium metaperiodate these agents gave unsatisfactory results (24). Osmium fixation can be reversed with sodium metaperiodate and antibody access is improved in epoxy resin sections by etching with hydrogen peroxide or sodium ethoxide. However, incubation with sodium metaperiodate is generally preferred for both etching and removal of osmium tetroxide thereby avoiding treatment with sodium ethoxide. This method gives higher immunolabelling densities, causes less damage to the sections and retains the best ultrastructural details (26).

2.6.2) Etching of LR White sections

For LR White sections etching is not absolutely necessary since LR White is hydrophilic. Therefore, unlike the heavily cross-linked hydrophobic epoxy plastics, water will pass into LR White sections as demonstrated by the swelling of the sections (9). Furthermore, retention of antigenicity in LR White sections is demonstrated by the short antibody incubation times necessary for successful immunolabelling (19) and immunolabelling patterns closely resembling those obtained on cryo-sections (18). Nevertheless, etching procedures have been shown to enhance the labelling of material embedded in LR White resins (2). Of the etching agents used with LR White embedded tissue, a combination of an alcohol solution of sodium hydroxide followed by sodium metaperiodate, gave optimal labelling with minimal background (2). Hydrogen peroxide treatment gave comparable labelling. However, the resulting background was often significantly higher. This increased background was probably due to the production of aldehydes by oxidation of the resin components. Free aldehyde groups can non-

specifically bind to antibodies. Pre-treatment with saturated, aqueous sodium metaperiodate and 0.1 M HCl, while showing increased labelling compared to that obtained with untreated sections, appeared to dissolve the section and render it unstable in the electron beam (2). Etching pre-treatments of LR White embedded tissue improves the accessibility for immunolabelling and thus is essential if maximal labelling of antigens is to be achieved. However, it is essential to examine each combination of antigen/antibody individually as different fixation and etching regimes may need to be tested for optimal results (2). It was suggested that by pre-treating of LR White sections by sodium ethoxide the antigen damaging effect of sodium ethoxide could be evaluated. This applies when it is assumed that sodium ethoxide treatment makes no difference to the immunolabelling efficiency on LR White sections. However, it was shown that for some antigens better immunolabelling was achieved with sodium ethoxide treatment of LR White sections (20). It is presumed that in such cases the fixation bonds are released by the action of sodium ethoxide. Therefore, when the same intensity of immunolabelling are observed for both sodium ethoxide-treated and untreated LR White sections it cannot be excluded that the reason is a combination of damage to the antigens and release of the fixation bonds (20).

2.6.3) Non-specific labelling on etched sections

One of the drawbacks of etching is the possible occurrence of non-specific immunolabelling. More than one aspect may be responsible for this unwanted labelling on etched sections including the type of etching solution and the antibodies. In addition, etching of the section may result in the retrieval of not all of the antigens present in the cell. This might be due to the different localisations and interactions of the antigen in the cell. Immunolabelling of eosinophil lysozyme in unetched LR White sections demonstrated the presence of lysozyme in pale cytoplasmic granules and specific granule

matrices with no labelling in the crystalloid of the specific granules. However, lysozyme may also be present in the crystalloid but in a masked form – bound to major basic protein or sulfated glycosaminoglycans. Etching with sodium metaperiodate produced a distinct alteration in labelling pattern, with matrix label decreasing and crystalloids becoming positive. This change could be interpreted as destruction of accessible matrix antigen with concomitant release of more resistant masked antigen in the crystalloid. However, this treatment also produced labelling of crystalloids by normal rabbit IgG suggesting non-specific binding (27).

2.6.4) Antigen retrieval from formaldehyde-fixed tissue

Interaction of fixative with amino acid side-groups causes certain antigens to be unavailable for binding to the antibody with immunolabelling techniques. However, it has been shown that these cross-linkages can be reversed by high temperature heating (120°C) or strong alkaline treatment. This observation formed the basis for the development of antigen retrieval techniques (28, 29). Furthermore, as a result of the breakage of formaldehyde-induced cross-links between antigens, the extraction of diffusible blocking proteins could also play a role in the achievement of better immunolabelling (30). Other treatments employed for the successful retrieval of antigens include "chemical antigen retrieval procedures" such as exposure to 0.1% Triton X-100 (31) and 0.5% SDS (32). Heat-induced antigen retrieval produces a large increase of immunolabelling only when the tissue is fixed in formaldehyde. When the tissue is fixed in glutaraldehyde or ethanol, insignificant or only weakly increased immunolabelling is shown upon heat-mediated antigen retrieval. With ethanol fixation, a decrease in immunolabelling could even be shown, which may be explained by the possible extraction of antigens from the poorly ethanol-fixed tissue during the heating process, or by heat-induced destruction of the poorly fixed antigens (33). Although the precise

mechanism responsible for the retrieval of antigens during high temperature heating is not known, it has been shown that the extreme temperatures achieved during heat-mediated retrieval of antigens is an important factor for the successful retrieval of antigens (34). Other factors that could play a role in heat-mediated antigen retrieval procedures include the exposure time and the type and pH of the buffer. There are indications that the chelation or precipitation of tissue-bound calcium ions (and other divalent metal ions) could be a critical step in salt-mediated antigen retrieval (26). The acidity/alkalinity of different types of buffers is shown to result in differences in the success of the retrieval of the antigen. In a study by Boon it was shown that when the pH of the microwave-retrieval solution was between pH 3.5 and 5, little immunolabelling was demonstrated. However, upon an increase in the pH of the buffer to between 5 and 6.5 an increase in immunolabelling occurred (13). Although various studies indicated a difference in the success of immunolabelling with a change in the pH, it seems as though the critical factor for the successful retrieval of the antigen is the achievement of specific minimal temperatures. With heating at 100°C and above, better immunolabelling results are achieved than heating at 90°C. Furthermore, prolonging the exposure time at lower temperatures does not necessarily compensate for the lower temperatures, with heating at 90°C for 10 minutes being more effective than heating at 60°C for 120 minutes (28, 29). Nevertheless, when the time of heating at 65°C was increased to 24 hours and compared to heating at 121°C for 15 minutes and 99°C for 40 minutes, the immunolabelling achieved was similar and even better to heating at the higher temperatures for shorter times (35). In a study by Shi *et al.* various different solutions at different temperatures were compared for their antigen retrieval efficiency. The order of efficiency of the antigen retrieval methods were as follows; Tris (65°C and 99°C) and EDTA (65°C) > EDTA (99°C) and commercial solution (99°C) > distilled water (65°C and 121°C), commercial solution (65°C and 121°C), citric acid (65°C, 99°C and 121°C),

urea (65°C and 121°C), EDTA (121°C) and Tris (121°C) > phosphate buffer (65°C, 99°C and 121°C) at 5% significance level. The difference among antigen retrieval in Tris (65°C), EDTA (65°C) and Tris (99°C) could not be statistically determined. It can be seen that irrespective of the temperature certain solutions can bring about better retrieval of antigens followed by increased immunolabelling. Comparing the efficiency of antigen retrieval in different buffer solutions at similar temperatures (microwave heating), 0.1 M Tris-HCl buffer, pH 9.5 containing 5% urea, 0.1 M Tris-HCl buffer, pH 9.5 without urea, and citrate buffer pH 6, for a panel of 34 antibodies, the Tris-HCl buffer containing urea was superior to the citrate buffer for 22 antibodies, in 12 cases the Tris-HCl buffer with urea was also superior to the Tris-HCl buffer without urea, in 12 cases the intensity was similar for all three antigen retrieval solutions, the staining obtained with Tris-HCl with urea was equal to or better than with pH 6 citrate buffer in all cases. This demonstrates that with antigen retrieval in solutions with a higher pH (8-10) better results are obtained for many antibodies (35). Also in routine sections of normal and pathological samples fixed in 10% buffered formalin, including EDTA-decalcified bone marrow biopsies, the higher pH solutions are superior to lower pH solutions. Immunolabelling were evaluated for 61 antibodies following heating in three different fluids including 0.01 M citrate buffer pH 6, 0.1 M Tris-HCl buffer pH 8, and 1 mM EDTA-NaOH solution pH 8. The sections underwent either three cycles of microwave treatment (5 minutes each) or pressurised cooking for 1-2 minutes. In comparison with the other fluids the EDTA-NaOH solution appeared to be superior in terms of both staining intensity and the number of marked cells (36). In a study employing a citrate buffer 10-50 mM, pH 8.5-9 equally good immunolabelling was obtained at all temperatures between 80°C and 100°C for only a short period of heating (30 minutes) (37), again demonstrating the superiority of the high pH citrate buffer that compensates for the lower heating temperatures.

A mechanism that could contribute to the retrieval of antigens and increase in immunolabelling efficiency upon heat-induced antigen retrieval is the restoration of electrostatic (coulombic) forces. It has also been suggested that prolonging the antibody incubation time from 10 minutes to 60 minutes represented an effective alternative to heat-induced antigen retrieval. Formaldehyde is an electrophilic substance that reacts with various functional groups of biologic macromolecules in a cross-linking fashion. When reacting with proteins the reactive hydrogen atoms are replaced by formaldehyde, which may be loosely (as Schiff's bases) or tightly bound (to form methylene bridges). Formaldehyde also disrupts hydrogen bonding and electrostatic interaction between amino acids and peptides, thus leading to changes in the secondary and tertiary structures within the target proteins and between the same and other proteins or tissue. All of these reactions are known to diminish the hydrophilic nature and thereby very likely reduce the net electrostatic charges of tissue antigens (38).

Microwave heating is the conventional heating method employed during heat-mediated antigen retrieval, however it is difficult to produce a controlled and uniform heating temperature with a microwave. By comparing the following heating methods a) microwave heating at 100°C for three periods of 5 minutes, b) autoclaving at 120°C for 10 minutes, c) pressurised boiling in a domestic pressure cooker at 120°C for 1 minute, 2 minutes or 5 minutes and d) boiling in an open glass jar for 15 minutes, no difference in immunolabelling was found among the four methods of heat treatment. There was, however considerable ultrastructural damage shown with the pressure cooker (39).

2.6.5) Antigen retrieval with proteolytic enzymes

Not only can antigens be retrieved by high temperature heating, but tissue digestion with proteolytic enzymes can also result in the retrieval of masked antigens for

immunolabelling procedures. Treatment with proteolytic enzymes is said to act by cleaving peptide bonds, thus uncovering antigenic sites from the proteinaceous web into which they have become woven during fixation (5, 40). Proteolytic digestion with 0.25% trypsin in PBS, pH 7.4 for 15 minutes to 60 minutes at 37°C was shown to successfully retrieve masked loricrin antigens (41). However, uncontrolled treatment with proteolytic enzymes can cause damage to the antigen and microwave antigen retrieval is therefore superior to enzyme digestion for various antigens. Microwave antigen retrieval methods (in a jar containing 250 ml of buffer irradiated for 8 minutes, i.e., 2 x 4 minutes) compared to enzymatic antigen retrieval methods including 0.1% trypsin for 20 minutes at 37°C and 0.5% protease VIII for 10 minutes at room temperature has shown superior immunolabelling for a panel of antibodies on formalin-fixed, paraffin-embedded sections. Furthermore, the microwave method seems to produce uniform immunostaining over large surface areas with no loss of morphological detail (40). For a panel of 60 antibodies it was shown that for only five of the antibodies proteolytic digestion with 0.05% protease XIV at 37°C for 5 minutes was superior to the conventional heat-based antigen retrieval procedure (36).

Not only are there differences in the antigen retrieval for different antigens but, there can also be differences for the same antigen in different subcellular pools. Suboptimal heat-mediated antigen retrieval, due to an inadequate heating period, can be a pitfall in immunolabelling because it may fail to reveal yet undiscovered sub-cellular pools of a particular antigen. Bcl-2 is one such antigen for which the retrieval of the antigen varies for the different sub-cellular Bcl-2 pools. Treatment for 10 minutes at 80-100°C in sodium-citrate buffer compared to 10 minutes of microwave heating, prolonged microwave heating (30 minutes at 100°C) or high pressure cooking (60 minutes at 130°C) improves cytoplasmic but not nuclear/chromosomal immunolabelling of human bcl-2 in

formaldehyde-fixed tissue sections. In contrast, these procedures restore the nuclear and mitotic chromosome-associated rat 68-86 bcl-2 antigen (42).

Not all antigens can be retrieved by using conventional antigen retrieval techniques. For these antigens the high temperatures (boiling) employed during these techniques may induce a negative result due to the destruction of the antigen. In these cases a lower-temperature heating treatment or a combination retrieval protocol (heat and enzyme digestion) may provide better results. In order to investigate the sensitivity of the antigens it is necessary to include control sections. A tissue section not treated by antigen retrieval is required to rule out any false-positive results or altered immunolabelling patterns (34).

2.6.6) Combination of etching and formaldehyde-fixed antigen retrieval

The following combinations of treatments have been compared for post-embedding immunolabelling a) no treatment, b) etching in H_2O_2 for 10 minutes, c) treatment with saturated aqueous sodium metaperiodate for 60 minutes, d) heating in H_2O at 91°C, e) heating in sodium citrate buffer at 91°C, f) heating in EDTA at 91°C, g) etching in H_2O_2 for 10 minutes followed by heating in sodium citrate buffer at 91°C, i) etching in H_2O_2 for 10 minutes followed by heating in EDTA at 91°C, j) treatment with sodium metaperiodate for 60 minutes followed by heating in H_2O at 91°C, k) treatment with sodium metaperiodate for 60 minutes followed by heating in sodium citrate buffer at 91°C and l) treatment with sodium metaperiodate for 60 minutes followed by heating in EDTA at 91°C. Only for heating in EDTA, no other technique, pre-treatment, or antigen retrieval procedure significantly improved immunolabeling with anti-AA amyloid. Furthermore, different types of fixatives appear to have little effect on EDTA retrieval. When immunolabelling with anti-transthyretin was investigated, only the combined pre-

treatment with H_2O_2 and EDTA and sodium metaperiodate and EDTA, was shown to significantly improve the immunolabelling (43).

2.7) Immunolabelling of the H-subunit and L-subunit of ferritin

2.7.1) Characteristics of the H-subunit and L-subunit monoclonal antibodies

Ferritin is structurally and immunologically very complex and the antigenic determinants are highly repetitive on the outer surface of the ferritin protein shell (44). The ferritin protein shell consists of 24 subunits of two different types, the H-subunit and the L-subunit. These two subunits, although they share only 55% amino acid sequence homology, fold into similar three-dimensional structures. However, the H-subunit has a more relaxed secondary structure than the L-subunit (45). On the outer surface of each of the 24 subunits a limited number of antigenic determinants at a short distance from one another are available for antibody binding. However, due to the large size and overlapping nature of the antigenic sites and the compact structure of the subunit, only one antigenic site can be occupied by an antibody at a time (46, 47). It seems also that these antigenic determinants are rather topographical than sequential since antibody binding is highly reduced and in some cases disappears after denaturation of ferritin (46). This is due to the complex quaternary structure of the ferritin protein shell, which results in the formation of antigenic determinants by sequences belonging to different subunits. Furthermore, can amino acid changes at a site distant from the antibody-binding site influence antibody binding (46). Nevertheless, certain antigenic determinants are insensitive to denaturation or renature easily indicating that certain antigenic determinants are mostly constituted of sequential amino acids (48).

Although the antigenic determinants of the ferritin subunits are similar resulting in the highly repetitive immunogenicity of ferritin, differences are shown to occur with certain

combinations of subunits in the ferritin protein shell. For some isoferritins, with only slightly different subunit compositions, major conformational differences are displayed. Conformational changes can result in masking or internalisation of antigenic determinants from one subunit and may also result in exposing new antigenic determinants or formation of new antigenic determinants by certain subunit interactions. In individual isoferritins with known H-subunit and L-subunit contents the measured immunoreactivities for H-subunit and L-subunit specific monoclonal antibodies reflected the relative contents of the two subunits. However, for certain isoferritins such as L-subunit rich isoferritins the H-subunit content is underestimated and in H-subunit rich isoferritins the H-subunit content is overestimated (47). This demonstrates that the H- and L-subunits are probably not randomly distributed in the protein shell (48).

The H-subunit and L-subunit contain common antigenic determinants that are non-specific for the subunits, as well as specific antigenic determinants – resulting in the elicitation of monoclonal antibodies that can discriminate between the H-subunit and the L-subunit. The non-specific antibodies can either bind to the different subunits with similar affinity or to the different subunits, but not with equal affinity. As a result of these differences in the antigenic determinants of the H-subunit and L-subunit, large immunological differences are seen in some tissue ferritins (47). Not all ferritins are immunologically the same. It has been shown that heart ferritin (rich in H-subunits) is immunologically more heterogenous than liver ferritin (rich in L-subunits) resulting in the elicitation of populations of antibodies with different specificities (48).

Murine monoclonal antibodies specific for the human H-subunit and L-subunit of ferritin is produced by Dr Paolo Arosio from the Department of Science and Biomedical Technology, University of Milan, Italy, and commercially available through Ramco

Laboratories, Inc., Stafford, Texas, United States of America. The monoclonal antibody specific for the H-subunit, RH02 an IgG1, is elicited in mice by recombinant H-subunit ferritin and does not cross-react with the L-subunit (Ramco Laboratories, Inc., Stafford, Texas, United States of America). The H-subunit monoclonal antibody RH02 has specificity analogous, but a higher affinity, than the previously generated 2A4 (44). The monoclonal antibody 2A4 was elicited by human heart ferritin consisting of 95% H-subunits and 5% L-subunits. Human heart ferritin contains at least one antigenic site, which is not present in human liver ferritin (44). The monoclonal antibody specific for the L-subunit, LF03 an IgG2B, is elicited by human liver ferritin and does not cross-react with the H-subunit (Ramco Laboratories, Inc., Stafford, Texas, United States of America).

In the present study, with ultrastructural visualisation of the immunolabelled sections, ferritin was seen as free ferritin molecules, clusters of ferritin molecules and as haemosiderin (denatured form of ferritin). Ferritin (containing a substantial amount of iron) is visible with the transmission electron microscope since iron is electron dense. Depending on the amount of iron contained by ferritin the ferritin particle will appear more or less electron dense. In most clusters the particles appeared larger and more electron dense than the free ferritin molecules and were thus of the iron-rich variety.

In this study it was found that the monoclonal antibodies only bind to the relatively iron-poor ferritin molecules and not to the more iron-rich electron dense ferritin particles. These findings might be explained by a change in the immunoreactivity of the H-subunit and L-subunit upon iron loading of the ferritin molecule. This is supported by a previous study which has shown that upon iron loading of cells, changes in the antigenicity of the surface of the protein shell occur which may be unrelated to subunit

composition (49). It has furthermore been suggested that these surface changes of the ferritin protein shell are associated with the formation of clusters of ferritin and subsequently haemosiderin (50).

It has been published that ferritin present in the nucleus of cells are H-subunit rich (51) and a specific pathway has been shown for the translocation of cytoplasmic H-subunit rich ferritins, but not cytoplasmic L-subunit rich ferritins, into the nucleus (52). In the present study immunolabelling was seen for both the H-subunit and the L-subunit monoclonal antibodies in the nucleus of macrophages and the cells of the erythron. It is therefore suggested that the ferritins which are found in the nucleus do contain a substantial amount of L-subunits. The presence and ratio of these subunits in the nucleus have not been quantified in the present study.

2.7.2) Secondary antibody gold-conjugate

Localisation of antigens with the electron microscope is achieved by a secondary antibody conjugated to a gold particle. This technique was used for the first time in 1971 by WP Faulk and GM Taylor – "An immunocolloid method for the electron microscope". They reported the adsorption of various primary antisera to particles of colloidal gold and the use of such antibody-gold complexes for direct electron microscopic localisation of surface antigens of Salmonella (53). Particles of colloidal gold can adsorb various proteins such as antibodies. Conjugation of antibodies to the gold particles occurs without any significant loss or alteration of the biological activity of the antibodies (54). Furthermore, colloidal gold does not display spontaneous affinity for ultrathin sections of resin-embedded tissues minimizing background labelling (54). Due to the high atomic number of gold, these gold-conjugates are of very high electron density providing improved spatial resolution and facilitating multiple labelling and

quantification of immunolabelling (53). Quantification of immunolabelling is made possible by the particulate nature of the gold-conjugate, which facilitates the counting of these markers. These colloidal gold particles are spherical in shape and can be prepared in sizes from 1 to 25 nm. Particles of 5-15 nm in size are excellent for post-embedding immunolabelling, particles of smaller size, such as 1 nm, must, however, be silver enhanced to be visible by the electron microscope (54). Antibodies bind to gold particles through the interaction of charges at the surface of the particle with those of the antibody through electrostatic Van der Waals forces, as well as more complex interactions (54). Colloidal gold is a hydrophobic sol formed by electron dense negatively charged particles with the stability of the colloidal gold in water maintained by electrostatic repulsion. However, when the particles reach a critical distance from one another it can result in flocculation leading to cohesion and precipitation of the colloid. This occurs in the presence of electrolytes that lead to reduction of the electrostatic repulsion. Binding of antibodies prevent flocculation by their association with the sol to form a shell around each particle (54).

With conjugation of the secondary antibody to colloidal gold the indirect technique of immunolabelling can be employed. In the first step the primary antibody is used in its native form to generate a specific antigen-antibody complex. In the second step this primary antibody complex becomes the target of the secondary antibody that is tagged with gold particles. With the use of this indirect technique of immunolabelling amplification of the signal can be achieved (54).

2.7.3) Non-specific binding of antibodies to the section

With post-embedding immunolabelling on resin sections, non-specific binding of the antibodies can occur to either the resin itself, non-specific reactive sites on the tissue or

to bio-molecules resembling the antigen. Non-specific binding to the resin and the embedded tissue may be due to the presence of chemical groups that react with the antibodies such as the presence of oppositely charged groups between the antibody and the embedded tissue (55). To reduce this non-specific labelling, blocking agents are used to prevent the reactive sites on the resin or tissue from binding to the antibodies. These blocking agents can include bovine serum albumin (BSA), ovalbumin, foetal calf serum, a mixture of Tween-20 and 0.5 M NaCl and a gelatine-containing buffer. Blocking agents are added to the antibody diluting and rinsing buffers (55, 56). However, the type of non-specific labelling caused by bio-molecular structures resembling the antigen is not blocked by BSA, but non-specificity caused by reactive groups in the resin or by electrostatic attraction between the tissue and the antibodies may be blocked. BSA molecules bind to reactive sites on the sections and occupy them, when the section is blocked before the introduction of the antibodies binding of BSA is non-competitive, by diluting the antibodies in a BSA-solution the BSA molecules will compete with the antibodies for binding with the reactive sites on the section, the use of a considerable excess of BSA compared to antibodies will make the antibodies loose the competition, and the non-specific labelling can be significantly reduced. Furthermore, with fixation in FA or GA, free aldehyde groups may remain after fixation. These free aldehyde groups may react with the antibody resulting in non-specific immunolabelling. This applies especially to GA. Due to the presence of two aldehyde groups, one end can be bound to cellular constituents with the other end free to react with the antibody. Blocking of these free aldehyde groups can be achieved with any small molecular-weight compound containing an amino group such as glycine, but other quenching agents can also be used such as ammonium chloride and sodium borohydride (7).

LR White is a hydrophilic resin and therefore displays a low attraction for other hydrophilic substances such as antibodies. This results in the reduction of non-specific binding of antibodies to the resin of the LR White section (9). However, for each antigen/antibody combination the possible non-specific binding to the resin should be investigated. By comparing non-specific labelling between epoxy sections and LR White sections, the epoxy sections normally give higher background labelling than LR White sections. The monomers producing polymerised epoxy resin are chemically reactive and may react with proteins. Antibodies can be covalently linked to the surface of the epoxy section, to un-polymerised epoxy resin and to end groups of polymer chains in the section. The chemical qualities of the monomers of LR White resin are different, the monomers are polymerised because their carbon-carbon double bonds have reactivity towards the radicals originally created by the initiator. The radicals produced through the polymerisation are usually terminated and even if they had reactivity against proteins they would not be capable of linking antibodies to the LR White section. Un-polymerised LR White monomers do not have special reactivity for proteins. Nevertheless, blocking procedures with at least 5% BSA are recommended for both epoxy and LR White sections. Both epoxy sections and LR White sections display non-specific binding but to a different degree and blocking of this non-specific binding under similar conditions are different for these two resins. With different BSA concentrations and lengths of the pre-incubation step varying between 0 and 4 hours, results show that the non-specific labelling on the resin decreases significantly when the concentration of BSA or the length of the pre-incubation step is increased. The non-specific labelling is usually higher on the epoxy resin than on the LR White resin when using the same conditions with respect to BSA, but when the pre-incubation step lasts 4 hours the non-specific labelling is somewhat lower on the epoxy resin than on the LR White resin. The specific labelling for the antibodies decreases slightly when the concentration of BSA and incubation time

increase. This is probably due to steric hindrance performed by BSA molecules on the section (55).

A major source of non-specific binding is a property of the colloidal gold itself. Adsorption of proteins to colloidal gold involves a combination of electrostatic and hydrophobic interactions and the degree to which a particle is coated is likely to be a function of the protein used. This explains why, for example, only small additions of antibodies are required for a gold preparation to reach saturation in the flocculation test, yet it is necessary to store the probe in a buffer containing an excess of unrelated protein (such as bovine serum albumin). However, even in the presence of excess BSA, it was demonstrated that such probes are capable of interacting with cell components and of generating non-specific background in immunolabelling experiments. Nevertheless, a substantial reduction in non-specific binding was shown when fish gelatine was substituted for bovine serum albumin as a stabilising agent or simply when fish gelatine was added to the probe buffer. This probably indicates that fish gelatine acts to coat a greater portion of the colloidal gold particle than either the antibody or bovine serum albumin. Furthermore, can the method used to prepare the colloidal gold particles also affect the non-specific binding properties of the resulting gold probes. White phosphorus and borohydride gold probes gave significantly lower levels of background staining than did the citrate-tannic acid gold probe. These differences may be due to different groups retained on the surface of the gold particles. The effectiveness of fish gelatine as a stabiliser for citrate-tannic acid probes is probably due to a greater affinity of tannic acid for gelatine than bovine serum albumin, preventing further interaction with other cell components (56).

3) Experimental evaluation of the ultrastructural immunolocalisation technique for the H-subunit and L-subunit of ferritin

The following aspects of the immunolocalisation technique for the H-subunit and L-subunit of ferritin were evaluated:

- The affinity of the H-subunit and L-subunit monoclonal antibodies for their respective recombinant H-ferritin and L-ferritin proteins.
- The cross-reactivity of the H-subunit monoclonal antibody toward the recombinant L-ferritin protein and the cross-reactivity of the L-subunit monoclonal antibody toward the recombinant H-ferritin protein.
- The effect of fixation and dehydration on the binding of the monoclonal antibodies to their respective recombinant ferritin proteins.
- The non-specific binding of the gold-conjugate secondary antibody to the sections.
- The non-specific binding of the monoclonal antibodies to the resin and different blocking procedures in order to reduce the non-specific binding.
- The effects of different preparations on immunolabelling:
    - Investigation of the effect of different antigen retrieval procedures on immunolabelling.
    - Investigation of the effect of different polymerisation procedures on immunolabelling.

3.1) The affinity of the H-subunit and L-subunit monoclonal antibodies for their respective recombinant H-ferritin and L-ferritin proteins

The affinities of the H-subunit and L-subunit monoclonal antibodies to their respective recombinant H-ferritin and L-ferritin proteins and the cross-reactivity of the H-subunit

monoclonal antibody toward the recombinant L-ferritin protein and the cross-reactivity of the L-subunit monoclonal antibody toward the recombinant H-ferritin protein were investigated with an ELISA.

Materials

1) Multiwell Immuno plate, NUNC™, Maxisorp, cat. no. M9410 – 1CS, Sigma-Aldrich, Aston Manor, South Africa.

2) 0.05 M sodium bicarbonate ($NaHCO_3$) buffer, pH 9.6, Sodium Bicarbonate, cat. no. 6329, Merck Chemicals (Pty) LTD., Germiston, South Africa.

3) Recombinant H-ferritin protein (RHF) and recombinant L-ferritin protein (RLF), Ramco Laboratories, Inc., Stafford, Texas, United States of America.

4) Phosphate buffered saline – 20 mmol/l sodium phosphate buffer, 0.15 mol/l sodium chloride. Two stock solutions were prepared – 20 mmol/l Na_2HPO_4, 0.15 mol/l NaCl and 20 mmol/l $NaH_2PO_4 \cdot H_2O$, 0.15 mol/l NaCl. The $NaH_2PO_4 \cdot H_2O$ stock solution was added to the Na_2HPO_4 stock solution to pH 7.4. Sodium chloride, SigmaUltra, cat. no. S7653, Sigma-Aldrich, Aston Manor, South Africa. di-Natriumhydrogenphosphate, cat. no. 6586, Merck Chemicals (Pty) LTD., Germiston, South Africa, Natriumdihydrogenphosphate-1-hydrate, cat. no. 6346, Merck Chemicals (Pty) LTD., Germiston, South Africa.

5) BSA, Bovine Serum Albumin, Amersham Biosciences, cat. no. RPN 412 V, Separations Scientific, Randburg, South Africa.

6) Primary monoclonal antibodies, monoclonal antibody specific for the H-subunit of ferritin, RH02 at a concentration of 0.2 mg/ml, and the monoclonal antibody specific for the L-subunit of ferritin, LF03 at a concentration of 0.2 mg/ml, Ramco Laboratories, Inc., Stafford, Texas, United States of America.

7) Secondary antibody a – peroxidase-goat anti-mouse IgG + A + M (H + L), cat. no. 62-6420, Zymed Laboratories, Inc., Scientific Group, Midrand, South Africa.

8) Secondary antibody b – anti-mouse IgG (whole molecule) peroxidase conjugate, cat. no. A 9044, Sigma-Aldrich, Aston Manor, South Africa.

9) Substrate solution – 30 mM citrate acid ($C_6H_8O_7$), cat. no. C0759, Sigma-Aldrich, Aston Manor, South Africa, 70 mM phosphate ($Na_2HPO_4 \cdot 2H_2O$), di-Natriumhydrogenphosphat-2-hydrat, cat. no. 6580, Merck Chemicals (Pty) LTD., Germiston, South Africa, pH 5 with concentrated NaOH, 1.5 mg/ml OPD (o-phenylenediamine), cat. no. P-1526, Sigma-Aldrich, Aston Manor, South Africa, 0.2 mg/ml H_2O_2, urea hydrogen peroxide tablets, U-8879, Sigma-Aldrich, Aston Manor, South Africa.

Method

1) The ELISA plate was coated with 10 µg/ml of either the recombinant H-ferritin protein (RHF) or the recombinant L-ferritin (RLF) protein in a 0.05 M sodium bicarbonate ($NaHCO_3$) buffer, pH 9.6 for 18 hours at 8°C.

2) The plate was rinsed 3 times with 280 µl of a 1% BSA (bovine serum albumin), phosphate buffered saline.

3) The plate was blocked with 280 µl of a 1% BSA, phosphate buffered saline for 1 hour at 37°C.

4) The plate was rinsed 3 times with 280 µl of a 1% BSA, phosphate buffered saline.

5) The plate was incubated with the primary antibody (1°), either rH02 (monoclonal H-ferritin antibody) or LF03 (monoclonal L-ferritin antibody), at different

concentrations, in 100 µl of 1% BSA, phosphate buffered saline for 2 hours at 37°C.

6) The plate was rinsed 6 times with 280 µl of a 1% BSA, phosphate buffered saline.

7) The plate was incubated with either one of the secondary antibodies (2°), horse radish peroxidase labelled anti-mouse IgG (1:200), in 100 µl of 1% BSA, phosphate buffered saline for 1 hour at 37°C.

8) The plate was rinsed 6 times with 280 µl of a 1% BSA, phosphate buffered saline.

9) The substrate solution (100 µl) was added to the plate.

10) The absorbances were measured at wavelengths of 450 nm and 630 nm. The absorbance values obtained at 630 nm were deducted from the absorbance values obtained at 450 nm.

Results

Table 1 and figure 1 show the results obtained for the affinity ELISA for the H-subunit and L-subunit monoclonal antibodies, at different concentrations, for their respective recombinant proteins.

Table 1. The absorbances obtained for the affinity ELISA for the H-subunit and L-subunit monoclonal antibodies at different concentrations for their respective recombinant proteins

Concentration mg/ml	Absorbance H-monoclonal	Absorbance L-monoclonal
0.002	1.056	0.417
0.001	0.869	0.379
0.0005	0.756	0.268
0.0004	0.755	0.232
0.0002	0.682	
0.0001	0.571	
0.00004	0.42	
0.00002	0.331	

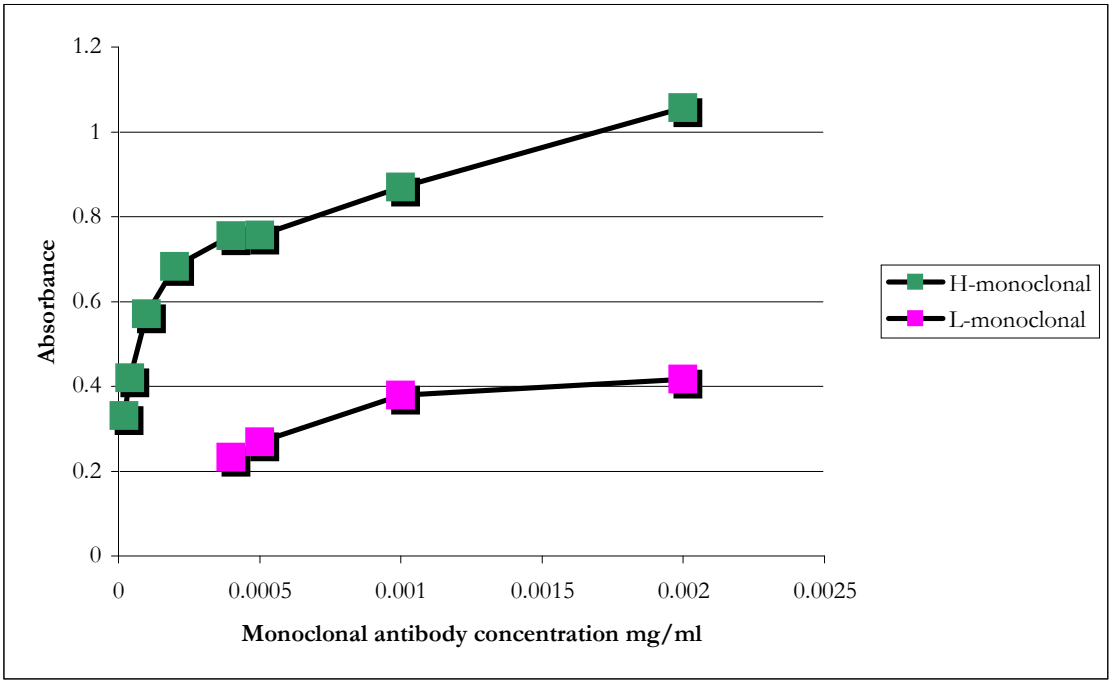

Figure 1. The absorbances obtained for the H-subunit monoclonal antibody (green) and the L-subunit monoclonal antibody (pink) at different concentrations for their respective recombinant proteins

The affinity of the H-subunit monoclonal antibody toward the recombinant H-ferritin protein was higher than the affinity of the L-subunit monoclonal antibody toward the L-ferritin recombinant protein. The affinity of the H-monoclonal antibody to the recombinant H-ferritin protein was shown to be about 2.5 times higher than the affinity of the L-monoclonal antibody to the recombinant L-ferritin protein.

3.2) The cross-reactivity of the H-subunit monoclonal antibody toward the recombinant L-ferritin protein and the cross-reactivity of the L-subunit monoclonal antibody toward the recombinant H-ferritin protein

Table 2 and figure 2 show the results obtained for the cross-reactivity ELISA for the H-subunit and L-subunit monoclonal antibodies for the recombinant proteins.

Table 2. The absorbances obtained for the affinity of the H-subunit and L-subunit monoclonal antibodies to their respective recombinant proteins and the cross-reactivities of the H-subunit and L-subunit monoclonal antibodies

	Absorbances
Coat RHF, 1° monoclonal H 1:100, 2°a 1:200	1.012
Coat RHF, 1° monoclonal H 1:100, 2°b 1:200	0.625
Coat RLF, 1° monoclonal H 1:100, 2°a 1:200	0.196
Coat RLF, 1° monoclonal H 1:100, 2°b 1:200	0.072
Coat RLF, 1° monoclonal L 1:100, 2°a 1:200	0.526
Coat RLF, 1° monoclonal L 1:100, 2°b 1:200	0.396
Coat RHF, 1° monoclonal L 1:100, 2°a 1:200	0.045
Coat RHF, 1° monoclonal L 1:100, 2°b 1:200	0.041

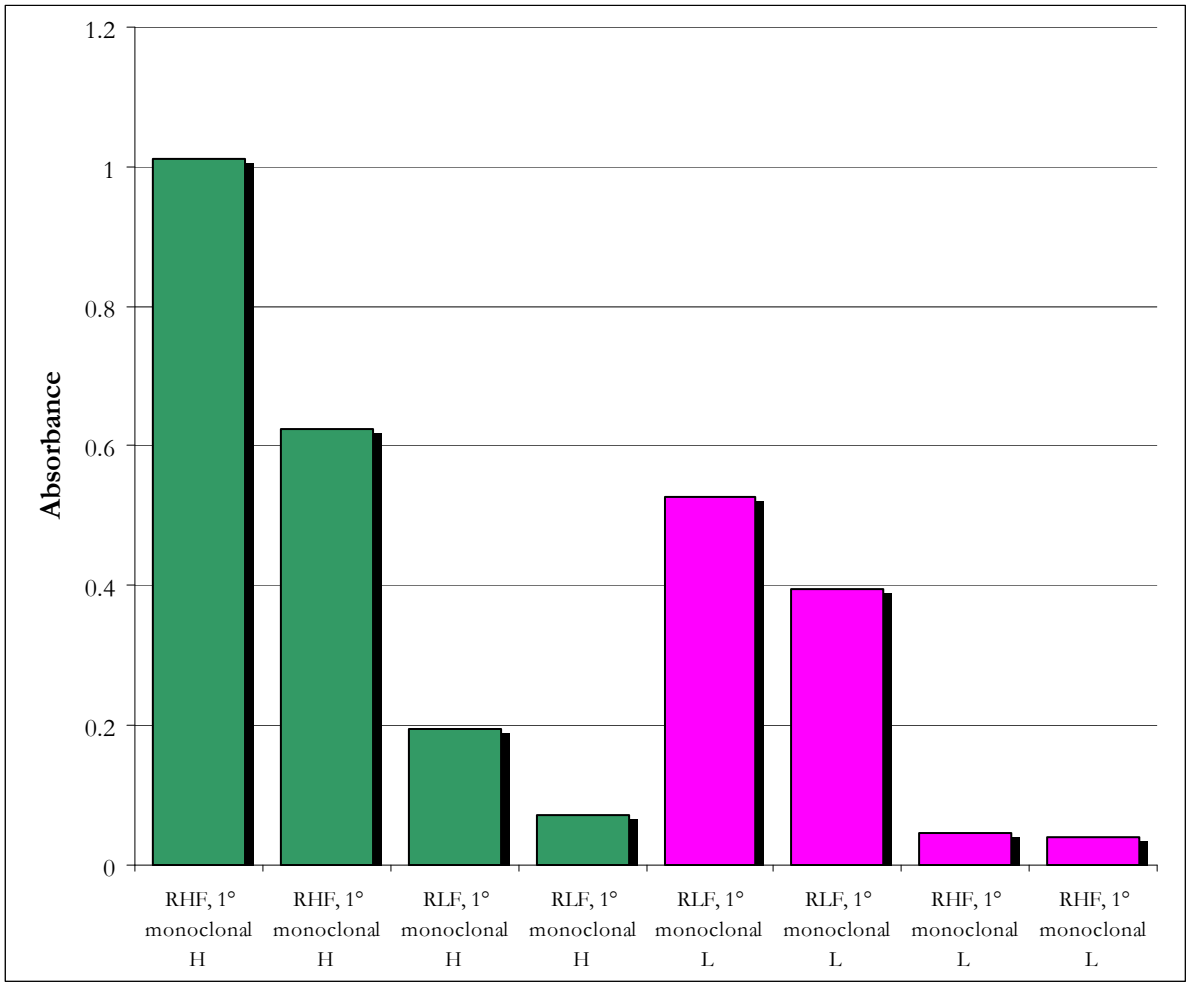

Figure 2. The absorbances obtained for the affinity of the H-subunit and L-subunit monoclonal antibodies to their respective recombinant proteins and the cross-reactivities of the H-subunit and L-subunit monoclonal antibodies

In figure 2 the first four columns (green) represents the binding of the H-subunit monoclonal antibody to the recombinant H-ferritin protein (first and second column) and recombinant L-ferritin protein (third and fourth column). Two different secondary antibodies were evaluated, secondary antibody a and secondary antibody b. Since different secondary antibodies will bind with different affinities to the primary antibodies. In the first column to the left and the third column, secondary antibody a was employed whereas in the second and fourth columns secondary antibody b was employed. The

absorbance of the first column (1.012) was the specific binding that the H-subunit monoclonal antibody had for the recombinant H-ferritin protein. The absorbance of the third column (0.196) was the cross-reactivity obtained for this combination of the H-subunit monoclonal antibody and secondary antibody to the recombinant L-ferritin protein. Thus, the H-subunit monoclonal antibody showed affinity for the recombinant L-ferritin protein, but 5 times less than that for the recombinant H-ferritin protein. For secondary antibody b, the absorbance of the second column (0.625) was the specific binding that the H-subunit monoclonal antibody had for the recombinant H-ferritin protein. The absorbance of the fourth column (0.072) was the cross-reactivity obtained for this combination of the H-subunit monoclonal antibody and secondary antibody to the recombinant L-ferritin protein. For this combination the H-subunit monoclonal antibody showed affinity for the recombinant L-ferritin protein, but 9 times less than that for the recombinant H-ferritin protein.

The last four columns (pink) of figure 2 represents the binding of the L-subunit monoclonal antibody to the recombinant L-ferritin protein and the recombinant H-ferritin protein. In the fifth column and seventh column secondary antibody a was employed whereas in the sixth column and eighth column secondary antibody b was employed. The absorbance of the fifth column (0.526) was the specific binding that the L-subunit monoclonal antibody had for the recombinant L-ferritin protein. The absorbance of the seventh column (0.045) was the cross-reactivity obtained for this combination of the L-subunit monoclonal antibody and secondary antibody to the recombinant H-ferritin protein. For this combination the L-subunit monoclonal antibody showed affinity for the recombinant H-ferritin protein, but 12 times less than that for the recombinant L-ferritin protein. For secondary antibody b, the absorbance of the sixth column (0.396) was the specific binding that the L-subunit monoclonal

antibody had for the recombinant L-ferritin protein. The absorbance of the eighth column (0.041) was the cross-reactivity obtained for this combination of the L-subunit monoclonal antibody and secondary antibody to the recombinant H-ferritin protein. For this combination the L-subunit monoclonal antibody showed affinity for the recombinant H-ferritin protein, but 10 times less than that for the recombinant L-ferritin protein. Therefore, the L-subunit monoclonal antibody showed negligible cross-reactivity towards the recombinant H-ferritin protein. Whereas, the H-subunit monoclonal antibody showed some cross-reactivity towards the recombinant L-ferritin protein but it was not critical.

3.3) The effect of fixation and dehydration on H-subunit and L-subunit monoclonal antibody binding to their respective recombinant H-ferritin and L-ferritin proteins

By coating an ELISA plate with the recombinant H-ferritin and L-ferritin proteins, followed by fixation and dehydration procedures similar to that as what will be used for tissue preparation, the effects of these processing steps on the binding of the monoclonal antibodies to their respective recombinant ferritin proteins can be investigated. Although, the effect of the prolonged exposure to 50°C is not investigated, it has been shown that this could have no effect. It is presumed that the fixative stabilises the antigen and prevents its destruction by exposure to such temperatures during polymerisation of the resin (57).

Additional materials

1) The fixative was prepared fresh immediately prior to the fixation step of the procedure. A 10% paraformaldehyde (Paraformaldehyde (Trioxymethylene), SPI Supplies, cat. no. 2615, Rick Loveland & Associates, Halfway House, South

Africa) solution in deionised H_2O was prepared fresh in a fume hood. The solution was heated to 60-70°C with constant stirring. Once the solution had reached the proper temperature stirring was continued for 15 minutes. At this point the solution was milky. One to two drops of 1 N NaOH was added, with stirring, until the solution cleared (7). The 0.15 M sodium phosphate buffer was prepared from two stock solutions. A 0.3 M Na_2HPO_4 stock solution (di-Sodium hydrogen phosphate Dihydrate, Fluka, BioChemika, Ultra, cat. no. 71643, Sigma-Aldrich, Aston Manor, South Africa) and a 0.3 M NaH_2PO_4 stock solution (Sodium dihydrogen phosphate Dihydrate, Fluka, Biochemika, MicroSelect, cat. no. 71505, Sigma-Aldrich, Aston Manor, South Africa). The 0.3 M NaH_2PO_4 stock solution was added to the 0.3 M Na_2HPO_4 stock solution to pH 7.25 immediately prior to the fixation step. This 0.3 M sodium phosphate buffer was then diluted 1:1 with the 10% freshly prepared formaldehyde stock solution and deionised H_2O. This was then followed by the addition of GA if necessary (Pure Glutaraldehyde 25% solution, E.M. grade, SPI Supplies, cat. no. 2607, Rick Loveland & Associates, Halfway House, South Africa).

2) Ethanol 99.9% Absolute A.R., Minema, Rick Loveland & Associates, Halfway House, South Africa.

3) Tween-20, Polyoxyethylenesorbitan monolaurate, Sigma for Molecular Biology, cat. no. P-9416, Sigma-Aldrich, Aston Manor, South Africa.

Method

1) The ELISA plate was coated with 10 µg/ml of either the recombinant H-ferritin protein (RHF) or the recombinant L-ferritin protein (RLF) in a 0.05 M sodium bicarbonate ($NaHCO_3$) buffer, pH 9.6 for 18 hours at 8°C.

2) The coating buffer containing either the recombinant H-ferritin protein or recombinant L-ferritin protein was removed from the plate.

3) The recombinant proteins were fixed with 100 µl of each of the following combinations; 4% formaldehyde; 4% formaldehyde, 0.05% glutaraldehyde; 4% formaldehyde, 0.1% glutaraldehyde; 4% formaldehyde, 0.5% glutaraldehyde in a sodium phosphate buffer for 1 hour at 8°C.

4) The plate was rinsed 1 time with 100 µl sodium phosphate buffer.

5) The plate was dehydrated as follows; 100 µl, 50% EtOH, 15 minutes at 8°C; 100 µl, 70% EtOH, 15 minutes at 8°C; 100 µl, 85% EtOH, 15 minutes at 8°C.

6) The plate was rinsed 3 times with 280 µl of a 1% BSA (bovine serum albumin), 0.05% Tween-20, phosphate buffered saline.

7) The plate was blocked with 280 µl of a 1% BSA, 0.05% Tween-20, phosphate buffered saline for 1 hour at 30°C.

8) The plate was incubated with the primary antibody (1°) either rH02 (monoclonal H-ferritin antibody) or LF03 (monoclonal L-ferritin antibody) in 100 µl of 1% BSA, phosphate buffered saline for 2 hours at 37°C, 1:100 dilution.

9) The plate was rinsed 6 times with 280 µl of a 1% BSA, 0.05% Tween-20, phosphate buffered saline.

10) The plate was incubated with the secondary antibody (2°), horse radish peroxidase labelled anti-mouse IgG (1:200) in 100 µl of 1% BSA, 0.05% Tween-20, phosphate buffered saline for 1 hour at 37°C.

11) The plate was rinsed 6 times with 280 µl of a 1% BSA, 0.05% Tween-20, phosphate buffered saline.

12) The substrate solution (100 µl) was added to the plate.

13) The absorbances were measured at wavelengths of 450 nm and 630 nm. The absorbance values obtained at 630 nm were deducted from the absorbance values obtained at 450 nm.

Results

Table 3 and figure 3 show the percentage of the optimal signal obtained for the binding of the H-subunit and L-subunit monoclonal antibodies to their respective recombinant proteins after different treatments.

Table 3. The percentage of the optimal signal obtained for the binding of the H-subunit and L-subunit monoclonal antibodies to their respective recombinant proteins after different treatments

Treatment number	Treatment	Percentage of optimal signal	
		RHF	RLF
1	4% FA	85%	118%
2	4% FA	85%	118%
3	4% FA, 0.05% GA	80%	79%
4	4% FA, 0.1% GA	71%	73%
5	4% FA, 0.5% GA	46%	60%
6	Dehydrate	93%	100%
7	4% FA, dehydrate	101%	146%
8	4% FA, 0.05% GA, dehydrate	87%	105%
9	4% FA, 0.1% GA, dehydrate	73%	118%
10	4% FA, 0.5% GA, dehydrate	54%	82%

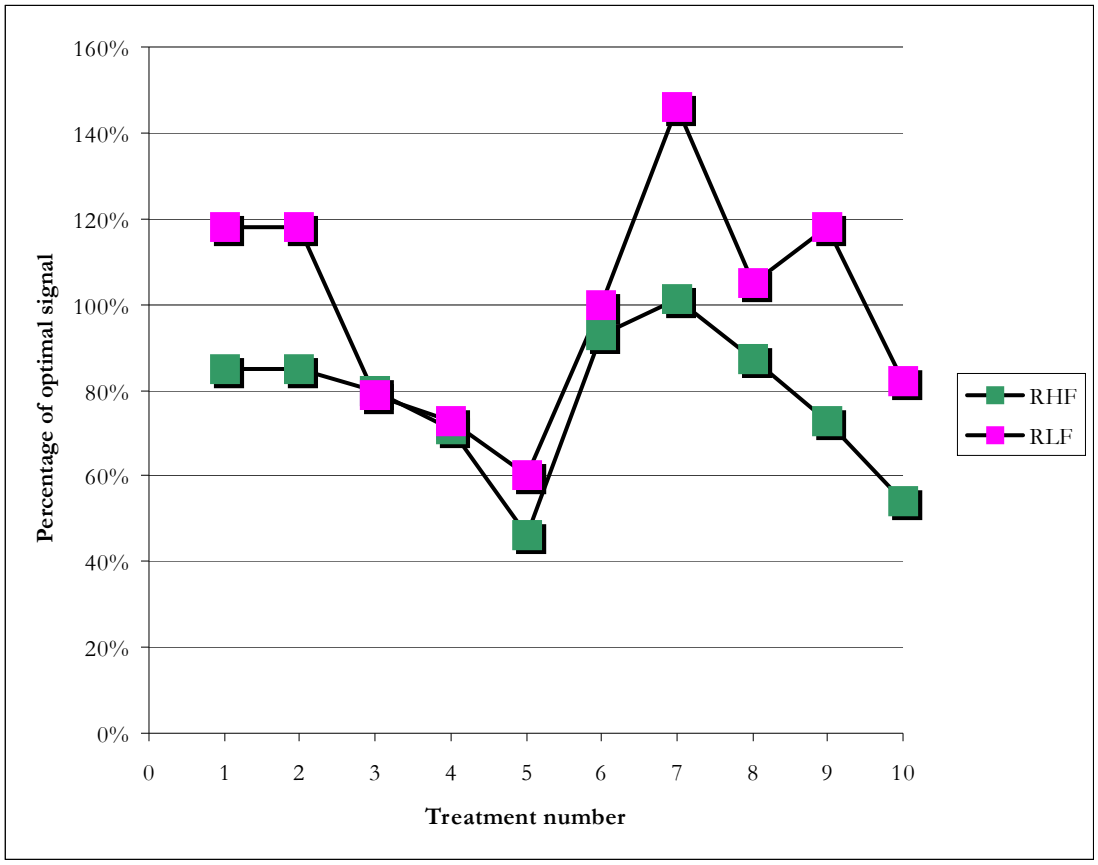

Figure 3. The percentage of the optimal signal obtained for the binding of the H-subunit and L-subunit monoclonal antibodies to their respective recombinant proteins after different treatments

In figure 3 for treatment 1 and 2 (fixation in 4% formaldehyde without dehydration) the affinity of the antibodies was shown to be influenced differently. For the H-subunit monoclonal antibody (green) there was a decrease in affinity to 85% whereas there was an increase in affinity to 118% for the L-subunit monoclonal antibody (pink). Treatment of the recombinant proteins with 4% formaldehyde and 0.05% glutaraldehyde (treatment 3), similar to what was used in the fixation of the bone marrow tissue, but still without dehydration, the affinity of the monoclonal antibodies were shown to be similarly decreased. A decrease to 80% was shown for the H-subunit monoclonal antibody (green) and a decrease to 79% was shown for the L-subunit monoclonal antibody (pink).

When the glutaraldehyde concentration was increased (treatment 4 and 5), both of the monoclonal antibodies were shown to have a decrease in affinity towards their respective recombinant ferritin proteins. However, the decrease for the H-subunit monoclonal antibody (treatment 5, green) was more than that of the L-subunit monoclonal antibody (treatment 5, pink) with a glutaraldehyde concentration of 0.5%. When the recombinant proteins were not fixed but only dehydrated (treatment 6) the affinity of the H-subunit monoclonal antibody (green) was shown to decrease (93%) whereas there was no difference shown for the affinity of the L-subunit monoclonal antibody (pink) from the optimal signal. Dehydration with ethanol could either have fixed the recombinant proteins, with or without changing the antigen, or extracted the recombinant proteins. With treatment 7 the recombinant proteins were fixed with 4% formaldehyde followed by dehydration with ethanol. No difference was shown for the affinity of the H-subunit monoclonal antibody (green), but a more pronounced increase was shown for the affinity of the L-subunit monoclonal antibody (pink) compared to when the recombinant L-ferritin was only fixed in 4% formaldehyde. With the addition of glutaraldehyde to the fixation solution (treatment 8, 9 and 10) followed by dehydration, a decrease was shown in the affinities for both the monoclonal antibodies to their respective ferritin proteins. However, it is different from that shown when the recombinant proteins were only fixed. The inclusion of dehydration could possibly have resulted in an additional fixation step and therefore an increase in the affinities of the monoclonal antibodies to their respective recombinant proteins.

3.4) Fixation of the core bone marrow tissue

With the suggestions found in the literature and the previous results the following method was used for the fixation, dehydration and embedding of the core bone marrow tissue.

Materials

1) Fixative consisting of 4% formaldehyde (FA), 0.05% glutaraldehyde (GA) in a 0.15 M sodium phosphate buffer. The fixative was prepared fresh immediately prior to the obtainment of bone marrow tissue. A 10% paraformaldehyde (Paraformaldehyde (Trioxymethylene), SPI Supplies, cat. no. 2615, Rick Loveland & Associates, Halfway House, South Africa) solution in deionised H_2O was prepared fresh in a fume hood. The solution was heated to 60-70°C with constant stirring. Once the solution had reached the proper temperature stirring was continued for 15 minutes. At this point the solution was milky. One to two drops of 1 N NaOH was added, with stirring, until the solution cleared (7). The 0.15 M sodium phosphate buffer was prepared from two stock solutions. A 0.3 M Na_2HPO_4 stock solution (di-Sodium hydrogen phosphate Dihydrate, Fluka, BioChemika, Ultra, cat. no. 71643, Sigma-Aldrich, Aston Manor, South Africa) and a 0.3 M NaH_2PO_4 stock solution (Sodium dihydrogen phosphate Dihydrate, Fluka, Biochemika, MicroSelect, cat. no. 71505, Sigma-Aldrich, Aston Manor, South Africa). The 0.3 M NaH_2PO_4 stock solution was added to the 0.3 M Na_2HPO_4 stock solution to pH 7.25 immediately prior to the obtainment of bone marrow tissue. This 0.3 M sodium phosphate buffer was then diluted 1:1 with the 10% freshly prepared formaldehyde stock solution and deionised H_2O. This was then followed by the addition of the GA (Pure Glutaraldehyde 25% solution, E.M. grade, SPI Supplies, cat. no. 2607, Rick Loveland & Associates, Halfway House, South Africa).

2) Ethanol 99.9% Absolute A.R., Minema, Rick Loveland & Associates, Halfway House, South Africa.

3) LR White Resin, medium grade acrylic resin, London Resin Company LTD., Rick Loveland & Associates, Halfway House, South Africa.

4) Gelatine capsules, SPI Supplies, cat. no. 2302, Rick Loveland & Associates, Halfway House, South Africa.

5) Nickel grids, 200 MESH Hexagonal grids, SPI Supplies, cat. no. 2480N, Rick Loveland & Associates, Halfway House, South Africa.

Method

1) A piece of core bone marrow was obtained during the biopsy procedure at the bedside of the patient and placed immediately in the fixative on ice.

2) The bone marrow tissue was fixed for 24 hours at 6°C whilst being rotated (TAAB rotator, Wirsam Scientific, Richmond, South Africa).

3) The bone marrow tissue was rinsed 3 times for 20 minutes with the sodium phosphate buffer at 6°C whilst being rotated.

4) The bone marrow tissue was dehydrated as follows 50% EtOH, 70% EtOH, 30 minutes each at 6°C whilst rotating followed by 85% EtOH, 2 times 15 minutes at 6°C whilst being rotated.

5) The bone marrow tissue was infiltrated with 1:1 85% EtOH:LR White mixture for 30 minutes at 6°C whilst being rotated. LR White dissolved in 85% EtOH but not in 80% EtOH.

6) The bone marrow tissue was infiltrated with LR White, 2 times 30 minutes at 6°C whilst being rotated.

7) The bone marrow tissue was placed in gelatine capsules in fresh LR White and was then polymerised, without any air bubbles, for 24 hours at 50°C.

8) The block of bone marrow tissue was sectioned and the sections were placed on nickel grids since copper grids can be oxidised during the immunolabelling procedures (54).

3.5) Outline of the method for the ultrastructural immunolocalisation of the H-subunit and the L-subunit of ferritin

An outline of the method for the immunolocalisation of the H-subunit and L-subunit of ferritin is presented here. The investigations that were done in order to determine the steps of the final method are presented in subsequent sections.

Materials

1) 8% $NaJO_4$, Sodium (meta) periodate, Fluka, Biochemika, Ultra, cat. no. 71859, Sigma-Aldrich, Aston Manor, South Africa.

2) Phosphate buffered saline – 20 mmol/l sodium phosphate buffer, 0.15 mol/l sodium chloride. Two stock solutions were prepared – 20 mmol/l Na_2HPO_4, 0.15 mol/l NaCl and 20 mmol/l $NaH_2PO_4 \cdot H_2O$, 0.15 mol/l NaCl. The $NaH_2PO_4 \cdot H_2O$ stock solution was added to the Na_2HPO_4 stock solution to pH 7.4. Sodium chloride, SigmaUltra, cat. no. S7653, Sigma-Aldrich, Aston Manor, South Africa, di-Natriumhydrogenphosphate, cat. no. 6586, Merck Chemicals (Pty) LTD., Germiston, South Africa, Natriumdihydrogenphosphate-1-hydrate, cat. no. 6346, Merck Chemicals (Pty) LTD., Germiston, South Africa.

3) 0.5% Glycine, Pharmacia Biotech, cat. no. 17-1323-01, AEC Amersham (PTY) LTD, Sandton, South Africa.

4) BSA, Bovine Serum Albumin, cat. no. RPN 412 V, Amersham Biosciences, Separations Scientific, Randburg, South Africa.

5) FBS, Fetal Bovine Serum, filtered and gamma irradiated, cat. no. 306, Highveld Biologicals (Pty) Ltd, Halfway House, South Africa.

6) Fish Gelatine (IGSS quality), cat. no. RPN 4160L/AB, Amersham Biosciences, Separations Scientific, Randburg, South Africa.

7) Tween-20, Polyoxyethylenesorbitan monolaurate, Sigma for Molecular Biology, cat. no. P-9416, Sigma-Aldrich, Aston Manor, South Africa.

8) Primary monoclonal antibodies, monoclonal antibody specific for the H-subunit of ferritin, RH02 and the monoclonal antibody specific for the L-subunit of ferritin, LF03 were obtained from Ramco Laboratories, Inc., Stafford, Texas, United States of America.

9) Secondary antibody, Anti-mouse IgG (whole molecule), gold conjugate, 10 nm, cat. no. G-7777, Sigma-Aldrich, Aston Manor, South Africa.

10) Glutaraldehyde, Pure Glutaraldehyde 25% solution, E.M. grade, SPI Supplies, cat. no. 2607, Rick Loveland & Associates, Halfway House, South Africa.

11) Uranyl acetate, SPI Supplies, cat. no. 2624, Rick Loveland & Associates, Halfway House, South Africa.

Method

1) Antigen retrieval step. All procedures were performed by placing the sections on a drop of the specific solution.

2) The sections were rinsed 3 times 5 minutes with phosphate buffered saline at room temperature.

3) First blocking step – the sections were blocked with 0.05% glycine in H_2O for 20 minutes at room temperature.

4) Second blocking step.

5) Incubation with the primary monoclonal antibodies in the second blocking solution.

6) The sections were rinsed 3 times 5 minutes with the second blocking solution at room temperature.

7) Incubation with the secondary antibody in the second blocking solution.

8) The sections were rinsed for 3 times 5 minutes with the second blocking solution at room temperature.

9) The sections were rinsed 5 minutes with phosphate buffered saline at room temperature.

10) The sections were fixed with 2% GA in phosphate buffered saline at room temperature.

11) The sections were rinsed 3 times 5 minutes with deionised H_2O at room temperature. A rinsing step in H_2O after the use of solutions containing phosphates is important to avoid the formation of artefactual electron dense deposits. The essential factors in the formation of electron dense deposits in tissues appear to be phosphate buffer, ethanol and uranyl acetate. The nature and intensity of the deposits seem to vary with the sequence of combination of these factors. Precipitation of phosphates has been observed in ethanol concentrations of 50% and more. Phosphates bind to uranyl ions due to a mordant action of the phosphate precipitates. Several different precipitate-forming processes may be involved: an interaction of phosphate and ethanol, of uranyl acetate and buffer and possibly an excess of unreacted aldehyde and uranyl acetate with or without phosphate (58).

12) To enhance the contrast of the sections, the sections were stained for 10 minutes with 0.3% uranyl acetate at room temperature.

13) The sections were dipped 15 times in 3 separate beakers with deionised H_2O.

14) The sections were then viewed with a Philips 301 transmission electron microscope.

3.6) Non-specific binding of the gold-conjugate secondary antibody

The possible non-specific binding of the gold-conjugate secondary antibody to the tissue section was investigated by incubating the tissue section with only the gold-conjugate secondary antibody – omitting the primary antibody from the incubation step (54).

Method

Steps were followed as for the outline of the immunolocalisation method with the specific solutions as follows:

1) The phosphate buffered saline was supplemented with 1% BSA.
2) The sections were incubated without the primary antibody for 2 hours in 1% BSA, phosphate buffered saline at room temperature.
3) The sections were incubated with the secondary antibody in the following dilutions 1:50, 1:150, 1:300 and 1:400 in 1% BSA, phosphate buffered saline for 1 hour at room temperature.

Results

No non-specific binding of the secondary antibody at any of the dilutions was shown. A dilution of 1:50 for the secondary antibody was used throughout.

3.7) Non-specific binding of the primary monoclonal antibodies to the resin

The investigation of the non-specific binding of the primary monoclonal antibodies was cumbersome. The primary monoclonal antibodies can bind non-specifically to the resin and to the tissue. The non-specific binding of the monoclonal antibody to the resin can be investigated more easily.

Method

Sections were made from clear polymerised resin without any embedded tissue. Steps were followed as for the outline of the immunolocalisation method with the specific solutions as follows. For the relevant rinsing steps, the blocking solution used for the section was also used for the rinsing steps.

1) One section was blocked with only phosphate buffered saline and 4 sections were blocked with 0.05% glycine in deionised H_2O for 1 hour at 30°C.
2) This was then followed by blocking with one of the following, phosphate buffered saline; 1% BSA in phosphate buffered saline; 1% FBS in phosphate buffered saline or 1% fish gelatine in phosphate buffered saline for 1 hour at 30°C.
3) The sections were incubated with the L-subunit monoclonal antibody at a final concentration of 1 µg/ml in each of the previous blocking solutions for 2 hours at 30°C.
4) The sections were incubated with the secondary antibody 1:50 in each of the previous blocking solutions for 1 hour at 30°C.
5) The sections were not stained with 0.3% uranyl acetate.

Results

The following number of gold particles was counted in a field of view at 22k magnification for each of the blocking procedures:

Only phosphate buffered saline	70 gold particles
Glycine and phosphate buffered saline	80 gold particles
Glycine and 1% BSA in phosphate buffered saline	25 gold particles
Glycine and 1% FBS in phosphate buffered saline	20 gold particles
Glycine and 1% fish gelatine in phosphate buffered saline	30 gold particles

With all these blocking procedures a substantial amount of non-specific binding was shown for the L-subunit monoclonal antibody. However, blocking with glycine followed by 1% FBS in phosphate buffered saline showed the least non-specific binding. In a second attempt the FBS was increased to 10%. The primary antibody solutions that were employed included the following final concentrations, 1 µg/ml, 0.4 µg/ml and 0.2 µg/ml.

Results

1 µg/ml L-subunit monoclonal antibody	3 gold particles
0.4 µg/ml L-subunit monoclonal antibody	0 gold particles in the field of view with a few on the whole section
0.2 µg/ml L-subunit monoclonal antibody	0 gold particles in the field of view with a few on the whole section

The non-specific binding obtained with the inclusion of 10% FBS resulted in negligible binding. Similar results were obtained for the H-subunit monoclonal antibody.

3.8) Investigation of the effect of the antigen retrieval procedures on non-specific binding to the resin of the monoclonal antibodies

Method

1) Sections of clear polymerised resin without any embedded tissue were used.
2) Sections were incubated with each of the following antigen retrieval solutions 0.01 M sodium citrate buffer pH 6, 0.01 M sodium citrate buffer pH 7.88, 0.05 M Tris-HCl buffer pH 10, 0.05 M Tris-HCl buffer pH 3 and 0.01 M EDTA-NaOH solution pH 8 for 30 minutes at 85°C or autoclaved at 121°C.

3) The sections were cooled down for 10 minutes.

4) The sections were rinsed 3 times 5 minutes with phosphate buffered saline at room temperature.

5) The sections were blocked with 0.05% glycine for 15 minutes at room temperature.

6) The sections were blocked with 10% FBS in phosphate buffered saline for 1 hour at 30°C.

7) The sections were incubated with the primary L-subunit monoclonal antibody at a concentration of 0.4 µg/ml in 10% FBS in phosphate buffered saline for 2 hours at 30°C.

8) The sections were rinsed 3 times 5 minutes with 10% FBS in phosphate buffered saline at room temperature.

9) The sections were incubated with the secondary antibody 1:50 in 10% FBS in phosphate buffered saline for 1 hour at 30°C.

10) The sections were rinsed 3 times 5 minutes in 10% FBS in phosphate buffered saline at room temperature.

11) The sections were not stained for contrast.

Results

For all the antigen retrieval solutions the amount of non-specific binding was negligible.

3.9) Investigation of the effect of different antigen retrieval procedures on immunolabelling

No specific immunolabelling was shown for the L-subunit and H-subunit monoclonal antibodies at a final concentration of 0.4 µg/ml or 4 µg/ml. Various antigen retrieval

procedures were tried to enhance the specific immunolabelling of the H-subunit and L-subunit monoclonal antibodies.

Materials

1) 0.01 M sodium citrate buffer, pH 6. The following two solutions were prepared, 0.01 M sodium citrate, analytical reagent, Protea Laboratory Services (PTY.) LTD., Johannesburg, South Africa and 0.01 M citric acid, analytical reagent, Hopkin & Williams LTD, Chadwell Heath, Essex, England. The 0.1 M citric acid solution was added to the 0.1 M sodium citrate solution to a pH of 6.
2) 0.05 M Tris-HCl buffer, pH 10, TRIS-HCl, Tris(hydroxymethyl)aminomethane hydrochloride, cat. no. 1.08219.0100, Merck Chemicals (Pty) LTD., Germiston, South Africa.
3) 0.01 M EDTA-NaOH solution, pH 8, Ethylene Diamine Tetra-acetic Acid, UNIVAR®, Saarchem, Krugersdorp, South Africa.
4) 0.1% TritonX-100 in deionised H_2O, Triton®X-100 Solution, cat. no. 93443, Fluka, BioChemika, MicroSelect, Sigma-Aldrich, Aston Manor, South Africa.
5) 0.5% SDS in deionised H_2O, Sodium dodecyl sulphate, cat. no. 17-1313-01, Pharmacia Biotech, Separations Scientific, Randburg, South Africa.
6) 3% H_2O_2 in deionised H_2O, Perdrogen 30% by weight H_2O_2, Riedel-de-Haën, cat. no. 31642, Sigma-Aldrich, Aston Manor, South Africa.
7) 0.1% and 0.25% trypsin, Trypsin / Versene in phosphate buffered saline, cat. no. 205, Highveld Biologicals (Pty) Ltd, Halfway House, South Africa.
8) 8% $NaJO_4$, Sodium (meta) periodate, Fluka, Biochemika, Ultra, cat. no. 71859, Sigma-Aldrich, Aston Manor, South Africa.

Method

1) Different antigen retrieval procedures were tried including heat-mediated antigen retrieval and chemical antigen retrieval. The procedures for the heat-mediated antigen retrieval were as follows; 0.01 M sodium citrate buffer pH 6, 0.05 M Tris-HCl buffer pH 10 and 0.01 M EDTA-NaOH solution pH 8. The sections were placed in 500 µl of the antigen retrieval solutions in an eppendorff and autoclaved at 121°C. The procedures for the chemical antigen retrieval procedures were as follows; 8% $NaJO_4$ in deionised H_2O for 1 hour at room temperature, 0.1% Triton X-100 in deionised H_2O for ten minutes at room temperature, 0.5% SDS in deionised H_2O for 10 minutes at room temperature and 3% H_2O_2 in deionised H_2O for 10 minutes at room temperature. All procedures were performed by placing the sections on a drop of the specific solution.
2) The autoclaved sections were cooled down for 10 minutes before the next step.
3) The sections were rinsed 3 times 5 minutes with phosphate buffered saline at room temperature.
4) The sections were blocked with 0.05% glycine in deionised H_2O for 20 minutes at room temperature.
5) The sections were blocked with 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline for 1 hour at 30°C.
6) The sections were incubated with the primary H-subunit monoclonal antibody at a concentration of 4 µg/ml in 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline for 2 hours at 30°C.
7) The sections were rinsed 3 times 5 minutes with 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline at room temperature.

8) The sections were incubated with the secondary antibody 1:50 in 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline for 1 hour at 30°C.

9) The sections were rinsed 3 times 5 minutes in 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline at room temperature.

10) The sections were rinsed 5 minutes with phosphate buffered saline at room temperature.

11) The sections were fixed with 2% GA in phosphate buffered saline at room temperature.

12) The sections were rinsed 3 times 5 minutes with deionised H_2O at room temperature.

13) The sections were stained for 10 minutes with 0.3% uranyl acetate at room temperature.

14) The sections were dipped 15 times in 3 separate beakers with deionised H_2O.

Results

No sufficient specific immunolabelling was achieved for the H-subunit monoclonal antibody at a concentration of 4 µg/ml with any of the antigen retrieval procedures.

3.10) Specific immunolabelling of the monoclonal antibodies

Specific immunolabelling was achieved (but low) only when the concentration of the primary antibodies was increased to 20 µg/ml and the incubation period prolonged to 4 hours at 30°C for both the H-subunit and the L-subunit. No antigen retrieval procedures. Figures 4a and 4b.

In an attempt to further increase the specific immunolabelling various antigen retrieval procedures were tried again. An increase in specific immunolabelling was achieved with antigen retrieval in 8% $NaJO_4$ for one hour at room temperature. Figures 4c and 4d.

An additional antigen retrieval step was included after antigen retrieval in 8% $NaJO_4$ for one hour at room temperature to possibly increase the specific immunolabelling. The following combined antigen retrieval procedures were tried; 8% $NaJO_4$ for 1 hour at room temperature and autoclaving in 0.01 M sodium citrate buffer pH 6, 8% $NaJO_4$ for 1 hour at room temperature and autoclaving in 0.01 M EDTA-NaOH solution pH 8 and 8% $NaJO_4$ for 1 hour at room temperature and autoclaving in 0.05 M Tris-HCl buffer pH 10. For all these combined antigen retrieval procedures an increase in specific immunolabelling was achieved over and above that for the 8% $NaJO_4$ for 1 hour at room temperature antigen retrieval step. Figures 4e and 4f, figures 5a and 5b and figures 5c and 5d.

Various chemical antigen retrieval procedures and enzymatic antigen retrieval procedures were also tried. The chemical antigen retrieval procedures included treatment with 0.1% Triton X-100 and 0.5% SDS. The enzymatic antigen retrieval procedures included digestion with 0.1% trypsin and 0.25% trypsin. Figures 5e and 5f, figures 6a and 6b and figures 6c and 6d.

3.11) Antigen retrieval with sodium ethoxide

Treatment with different antigen retrieval methods resulted in an increase in the specific labelling of the H-subunit and L-subunit of ferritin. However, it was not satisfactory. Antigen retrieval in sodium ethoxide was tried in order to possibly increase the specific immunolabelling.

Materials

1) An 11% solution of sodium ethoxide was prepared as follows; add 11% NaOH in absolute anhydric ethanol. Anhydric ethanol was prepared by using molecular sieves (Molecular sieves, 3 Å, powder, undried, cat. no. 23,364-1, Sigma-Aldrich, Aston Manor, South Africa). The molecular sieves were activated at 250°C for at least 3 hours. Absolute ethanol was incubated with the molecular sieves at a concentration of 5% (w/v) at room temperature for 12 hours on a magnetic stirrer. This was repeated with a new batch of molecular sieves. The container was closed with parafilm and the final anhydric solution was decanted into dried (250° celcius) glassware (25).

Method

1) The sections were incubated in a 1:10 or 1:100 dilution (1% and 0.1% sodium ethoxide) of 11% NaOH in absolute anhydric ethanol at room temperature for 60 seconds.
2) The sections were rinsed for 5 minutes in 100% ethanol at room temperature.
3) The sections were rinsed for 5 minutes in 70% ethanol at room temperature.
4) The sections were rinsed for 5 minutes in 50% ethanol at room temperature.
5) The sections were rinsed 3 times 5 minutes with phosphate buffered saline at room temperature.

Results

No increase in the specific immunolabelling was shown for antigen retrieval in sodium ethoxide. The sodium ethoxide resulted in damage to the plastic sections.

3.12) Investigation of the effect of different polymerisation procedures on immunolabelling

In a final attempt to increase the specific immunolabelling of the H-subunit and L-subunit of ferritin, different polymerisation procedures were tried. These included heat polymerisation, catalytic polymerisation and UV-light polymerisation.

Methods

Heat polymerisation

1) After the final change of pure LR White the bone marrow was placed in pure LR White in a gelatine capsule and polymerised at 50°C for 24 hours.

Catalytic polymerisation

1) The polymerisation mixture was prepared as follows. 15 µl of accelerator was added to 10 ml of pre-cooled (4°C) LR White monomer. This mixture was stirred gently, careful not to introduce any air bubbles.
2) After the final change of pure LR White the bone marrow was placed in this mixture in a gelatine capsule.
3) Catalytic polymerisation is an exothermal reaction, therefore in order to ensure dissipation of the generated heat the capsules were placed in a pre-cooled solid aluminium block with drilled holes.
4) The gelatine capsules were left at 4°C for 24 hours for completion of the polymerisation process.

UV-light polymerisation

1) After the final change of pure LR White the bone marrow was placed in pure LR White in a gelatine capsule.
2) The gelatine capsules were placed in a wire rack inside a polystyrene box coated with aluminium foil in a freezer (-20°C).
3) The UV light source (360 nm) was placed on top of the box with a distance of 17 cm from the gelatine capsules.
4) The gelatine capsules were left in the freezer for 36 hours for completion of the polymerisation process. The temperature in the freezer increased to -10°C as a result of the heat generated by the lamp.

Results

No increase in specific immunolabelling was shown for any of the polymerisation procedures. Heat polymerisation was used throughout. Figures 6e and 6f.

3.13) The achievement of satisfactory immunolabelling

It was only with the use of new antibodies at a higher concentration that sufficient immunolabelling was achieved. These antibodies were received from Prof Paolo Arosio. The concentration of these antibodies was 1 mg/ml and was employed at a final concentration of 50 µg/ml. However, a concentration of 25 µg/ml also resulted in sufficient immunolabelling. Figures 7a and 7b. In order to investigate the possible non-specific binding of these antibodies to the resin immunolabelling was performed with an increase in the Tween-20 concentration. No difference in non-specific binding was shown for Tween-20 concentrations of 0.05%, 0.1%, 0.5% and 1%. Figures 7c-7f.

3.14) Discussion

In order to determine the differential expression of the H-subunit and L-subunit of ferritin, monoclonal antibodies specific to only the subunit of interest is of paramount importance. Ideally these two monoclonal antibodies should have similar affinities to their respective subunits and show no cross-reactivity to the other subunit. By using an ELISA-based method and recombinant H-ferritin and L-ferritin proteins these characteristics of the H-subunit and L-subunit monoclonal antibodies were investigated. It was shown that the H-subunit monoclonal antibody had an affinity towards the recombinant H-ferritin protein about 2.5 times more when compared to the affinity of the L-subunit monoclonal antibody towards the recombinant L-ferritin protein. Furthermore, it was shown that the H-subunit monoclonal antibody has a low affinity for the recombinant L-ferritin protein – 5 times less than that shown for the recombinant H-ferritin protein. Whereas, the L-subunit monoclonal antibody showed a very low affinity for the recombinant H-ferritin protein – 10 times less than that shown for the recombinant L-ferritin protein. The question remains as to whether these results can be applied to ultrastructural immunolocalisation. Since it was shown in the present study that only at concentrations of 20 µg/ml did specific immunolabelling occur for both the antibodies, but with the specific immunolabelling of the H-subunit much more problematic. It should be noted that with the ELISA method the affinity of these monoclonal antibodies was evaluated for their respective recombinant proteins. These recombinant proteins contain 100% H-subunits and 100% L-subunits respectively. Ferritin molecules containing 100% of either the H-subunit or the L-subunit are not likely to be encountered *in vivo* (59). Furthermore, it was shown that different combinations of the specific subunits in a ferritin molecule could influence the binding of the monoclonal antibodies to their respective subunits (47).

Not only do the H-subunit and L-subunit monoclonal antibodies display different affinities toward their respective recombinant ferritin proteins, but these affinities are influenced differently by fixation and dehydration. The effects of fixation and dehydration were investigated by an ELISA-based method. The binding of the monoclonal antibodies was influenced differently by the solutions that were used for fixation and dehydration of the bone marrow tissue. The H-subunit monoclonal antibody showed a decrease in affinity to 87% of the optimal signal whereas the L-subunit monoclonal antibody showed a small increase in affinity to 105% of the optimal signal. Therefore, the H-subunit monoclonal antibody epitope was more sensitive to the fixation and dehydration solutions. This could possibly be part of the reason why immunolabelling of the H-subunit was more difficult than immunolabelling of the L-subunit.

In the present study, the changes in the expression of the H-subunit, the changes in the expression of the L-subunit and the changes in the H-subunit/L-subunit ratio were investigated in subgroups of patients and not the absolute amount of the H-subunit relative to the absolute amount of the L-subunit. The obtainment of proper specific immunolabelling for both the H-subunit and the L-subunit of ferritin on the LR White sections was therefore the main objective.

No specific immunolabelling for either the H-subunit monoclonal antibody or the L-subunit monoclonal antibody at a final concentration of 4 µg/ml was obtained on the LR White sections. Different antigen retrieval procedures were tried in order to achieve specific immunolabelling. The procedures for the heat-mediated antigen retrieval were as follows; 0.01 M sodium citrate buffer pH 6, 0.05 M Tris-HCl buffer pH 10 and 0.01 M EDTA-NaOH solution pH 8. The sections were placed in 500 µl of the antigen retrieval

solutions in an eppendorff and autoclaved at 121°C. The procedures for the chemical antigen retrieval procedures were as follows; 8% $NaJO_4$ in deionised H_2O for 1 hour at room temperature, 0.1% Triton X-100 in deionised H_2O for ten minutes at room temperature, 0.5% SDS in deionised H_2O for 10 minutes at room temperature and 3% H_2O_2 in deionised H_2O for 10 minutes at room temperature. No specific immunolabelling of either the H-subunit monoclonal antibody or the L-subunit monoclonal antibody at a final concentration of 4 µg/ml was achieved with any of these antigen retrieval procedures.

The achievement of specific immunolabelling for the H-subunit and L-subunit of ferritin was problematic. The highest concentration (20 µg/ml) was used that was possible with the first batch of antibodies at a dilution of 1:10. With this concentration specific immunolabelling of the L-subunit monoclonal antibody was shown which could also be increased by various antigen retrieval solutions. Little specific immunolabelling was achieved for the H-subunit monoclonal antibody at a final concentration of 20 µg/ml with only a little increase in specific immunolabelling with different antigen retrieval solutions. One of the major obstacles with post-embedding immunolabelling is that immunolabelling arises from antigen only exposed on the surface of the section (included within an essentially two-dimensional space), therefore, a relatively large improvement in the volumetric retention of the antigen is required to show even a modest increase in surface restricted immunogold immunolabelling. Such an improvement is seen with cryo-immunolabelling where extraction due to dehydration and plastic embedding is completely eliminated (9). Nevertheless, an increase in the final monoclonal antibody concentration to 50 µg/ml resulted in specific immunolabelling for both the H-subunit and L-subunit of ferritin.

3.15) Final method for the ultrastructural immunolabelling of the H-subunit and L-subunit of ferritin

The final method for the immunolocalisation of the H-subunit and L-subunit of ferritin is presented here.

Method

1) The sections were incubated with 8% $NaJO_4$ in H_2O for 1 hour at room temperature. All procedures were performed by placing the sections on a drop of the specific solution.
2) The sections were rinsed 3 times 5 minutes with phosphate buffered saline at room temperature.
3) The sections were blocked with 0.05% glycine in H_2O for 20 minutes at room temperature.
4) The sections were blocked with 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline for 1 hour at 30°C.
5) The sections were incubated with the primary monoclonal antibodies 1:20 diluted in 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline for 4 hours at 30°C.
6) The sections were rinsed 3 times 5 minutes with 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline at room temperature.
7) The sections were incubated with the secondary antibody 1:50 in 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline for 1 hour at 30°C.
8) The sections were rinsed 3 times 5 minutes with 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline at room temperature.
9) The sections were rinsed 5 minutes with phosphate buffered saline at room temperature.

10) The sections were fixed with 2% GA in phosphate buffered saline at room temperature.

11) The sections were rinsed 3 times 5 minutes with deionised H_2O at room temperature.

12) To enhance the contrast of the sections, the sections were stained for 10 minutes with 0.3% uranyl acetate at room temperature.

13) The sections were dipped 15 times in 3 separate beakers with deionised H_2O.

14) The sections were then viewed with a Philips 301 transmission electron microscope.

Figure 4 a and b

Specific immunolabelling was achieved when the concentration of the primary antibody was increased to 20 µg/ml and the incubation period prolonged to four hours for the H-subunit and the L-subunit, respectively. No antigen retrieval procedures.

Figure 4 c and d

An increase in specific immunolabelling was achieved with antigen retrieval in 8% $NaJO_4$ for the H-subunit and the L-subunit, respectively.

Figure 4 e and f

An additional increase in specific immunolabelling was achieved when the 8% $NaJO_4$ treatment was followed by an additional antigen retrieval step with 0.01 M sodium citrate buffer, pH 6 (autoclave) for the H-subunit and the L-subunit, respectively. Scale bar 1 µm.

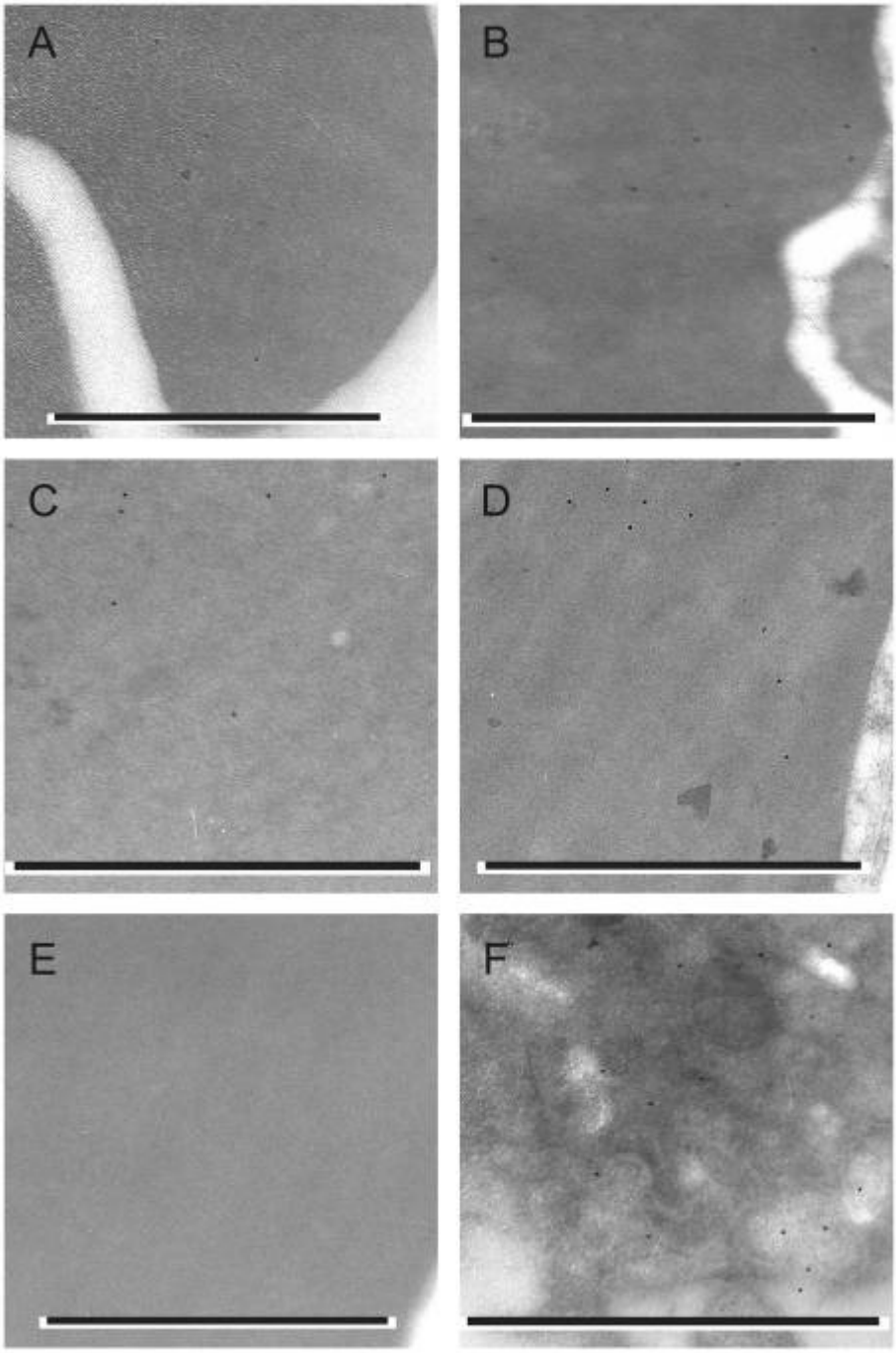
A
B
C
D
E
F

Figure 5 a and b

A further increase in specific immunolabelling was achieved when the 8% $NaJO_4$ was followed by an additional antigen retrieval step with 0.01 M EDTA-NaOH solution, pH 8 (autoclave) for the H-subunit and the L-subunit, respectively.

Figure 5 c and d

A further increase in specific immunolabelling was achieved when the 8% $NaJO_4$ treatment was followed by an additional antigen retrieval step with 0.05 M Tris-HCl buffer, pH 10 (autoclave) for the H-subunit and the L-subunit, respectively.

Figure 5 e and f

Antigen retrieval with 0.1% Triton X-100 for the H-subunit and the L-subunit, respectively. Scale bar 1 μm.

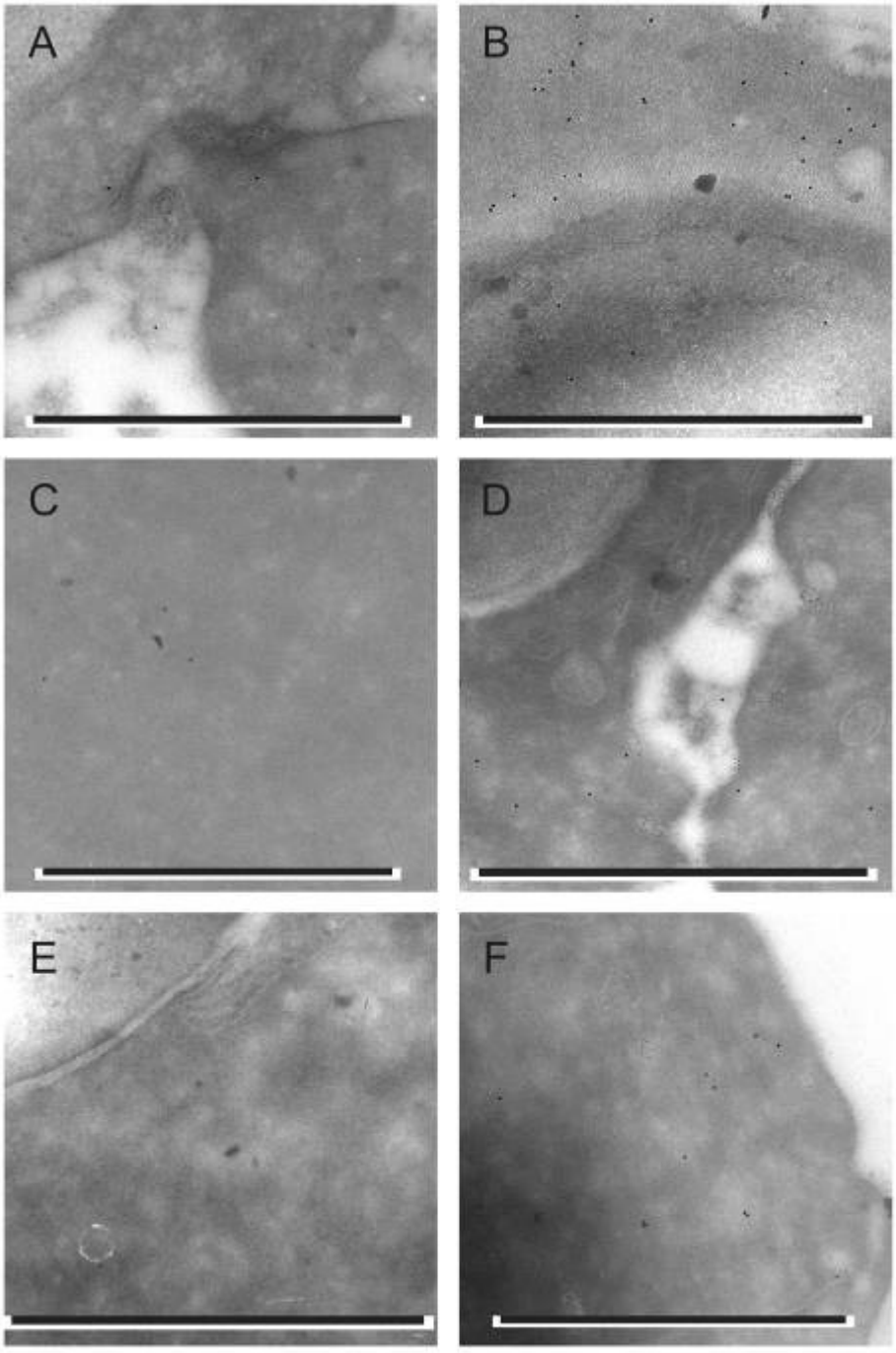
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Figure 6 a and b

Antigen retrieval with 0.5% SDS for the H-subunit and the L-subunit, respectively.

Figure 6 c and d

Antigen retrieval with 0.1% trypsin for the H-subunit and the L-subunit, respectively.

Figure 6 e

Polymerisation of LR White at 50°C for 24 hours.

Figure 6 f

Polymerisation of LR White with UV light at 10°C for 36 hours. Scale bar 1 µm.

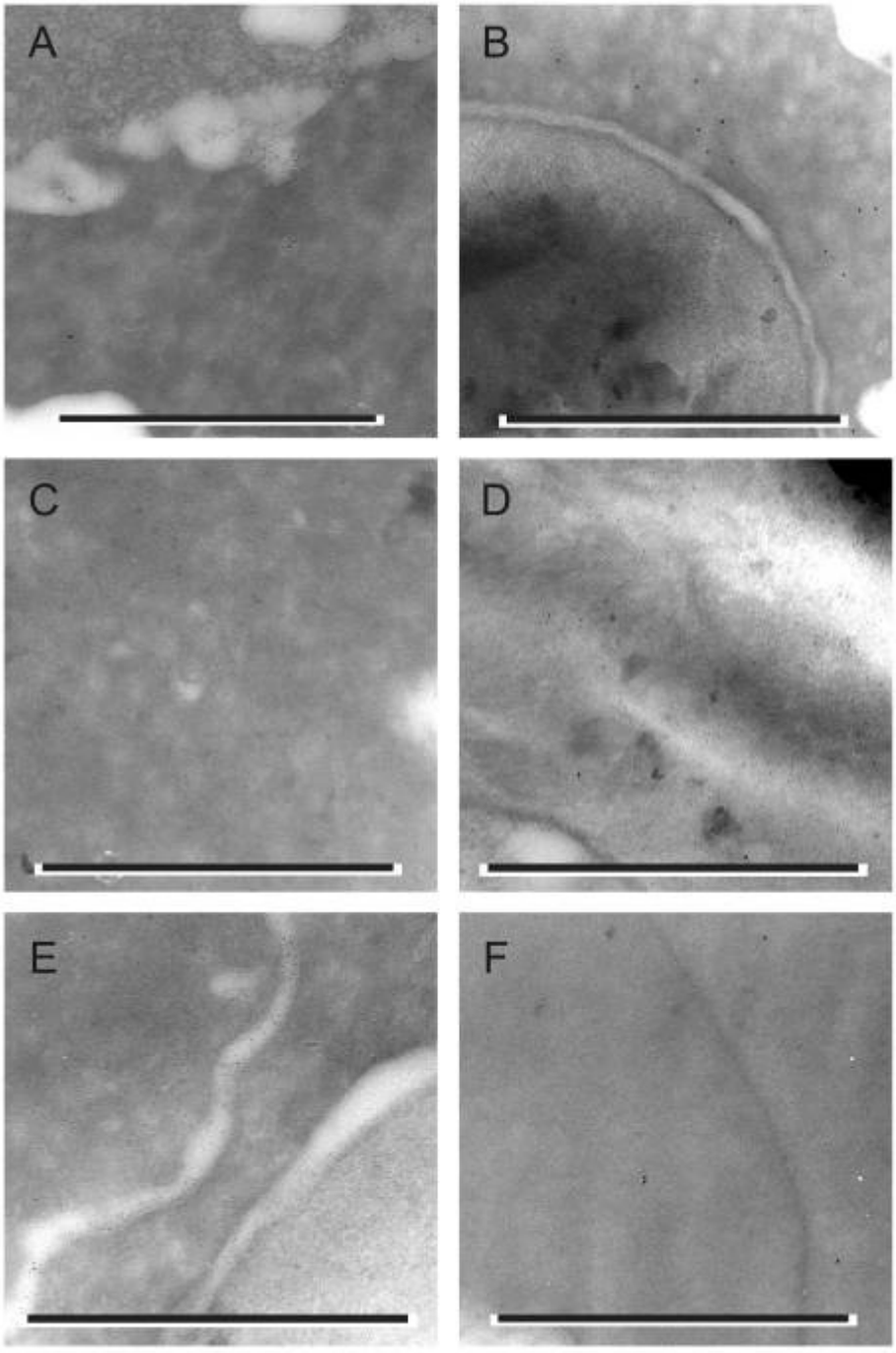
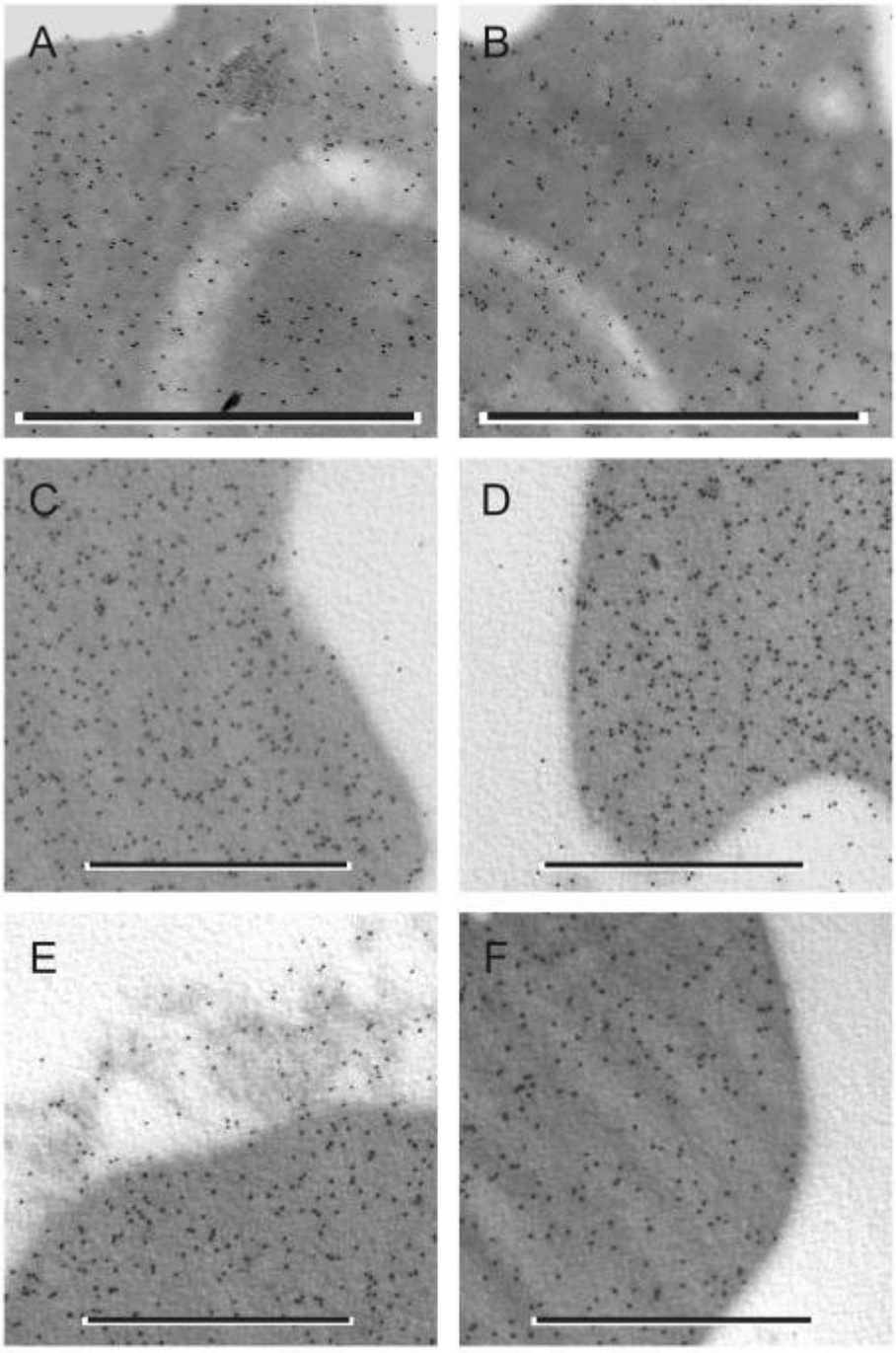

A
B
C
D
E
F

Figure 7 a and b

Immunolocalisation of the H-subunit and L-subunit with monoclonal antibodies at a concentration of 25 µg/ml, respectively.

Figure 7 c-f

The Tween-20 concentration was increased from 0.05%, to 0.1%, to 0.5% and finally to 1%. Scale bar 400 nm.


A
B
C
D
E
F

4) References

1) Glauert AM. Fixation, dehydration and embedding of biological specimens. In: Glauert AM editor. Practical methods in electron microscopy, vol. 3. Amsterdam: North-Holland Publishing Company; 1974. p. 1-207.

2) Ingram LT, Stenzel DJ, Kara UAK, Bushell GR. Localisation of internal antigens of *Plasmodium falciparum* using monoclonal antibodies and colloidal gold. Parasitology Research 1988; 74: 208-215.

3) Bendayan M, Nanci A, Kan FWK. Effect of tissue processing on colloidal gold cytochemistry. Journal of Histochemistry and Cytochemistry 1987; 35(9): 983-996.

4) Groos S, Reale E, Luciano L. Re-evaluation of epoxy resin sections for light and electron microscopic immunostaining. Journal of Histochemistry and Cytochemistry 2001; 49(3): 397-406.

5) Ring PKM, Johanson V. Immunoelectron microscopic demonstration of thyroglobulin and thyroid hormones in rat thyroid gland. Journal of Histochemistry and Cytochemistry 1987; 35(1): 1095-1104.

6) Newman GR, Hobot JA. Modern acrylics for post-embedding immunostaining techniques. Journal of Histochemistry and Cytochemistry 1987; 35(9): 971-981.

7) Oliver C. Fixation and embedding. In: Javois LC editor. Methods in Molecular Biology, vol. 34, Immunocytochemical Methods and Protocols. Totowa, New Jersey: Humana Press; 1994. p. 291-298.

8) Hobot JA, Newman GR. Strategies for improving the cytochemical and immunocytochemical sensitivity of ultrastructurally well-preserved, resin embedded biological tissue for light and electron microscopy. Scanning Microscopy Supplement 1991; 5: S27-S41.

9) Newman GR. Use and abuse of LR White. Histochemical Journal 1987; 19: 118-120.

10) Brorson S-H, Roos N, Skjorten F. Antibody penetration into LR-White sections. Micron 1994; 25(5): 453-460.

11) Brorson S-H. Antigen detection on resin sections and methods for improving the immunogold labelling by manipulating the resin. Histology Histopathology 1998; 13(1): 275-281.

12) Goght A. Use of LR White resin for post-embedding immunolabelling of brain tissue. Acta Anatomica 1992; 145: 327-339.

13) Boon ME. Microwave-antigen retrieval: the importance of pH of the retrieval solution for MiB-1 staining. European Journal of Morphology 1996; 34(5): 375-379.

14) Osamura RY, Itoh Y, Matsuno A. Applications of plastic embedding to electron microscopic immunocytochemistry and in situ hybridization in observations of production and secretion of peptide hormones. Journal of Histochemistry and Cytochemistry 2000; 48(7): 885-891.

15) Roth J. Post-embedding cytochemistry with gold-labelled reagents: a review. Journal of Microscopy 1986; 143(Pt 2): 125-137.

16) Kellenberger E. Learning about truth and biases through experience: section surface corrugation, protein denaturation, and staining. Microscopy Research and Technique 1998; 42: 33-42.

17) Ellinger A, Pavelka M. Post-embedding localization of glycoconjugates by means of lectins on thin sections of tissues embedded in LR White. Histochemical Journal 1985; 17: 1321-1336.

18) Timms BG. Postembedding immunogold labelling for electron microscopy using "LR White" resin. American Journal of Anatomy 1986; 175: 267-275.

19) Newman GR, Jasani B, Williams D. A simple post-embedding system for the rapid demonstration of tissue antigens under the electron microscope. Histochemical Journal 1983; 15: 543-555.

20) Brorson S-H. How to examine the antigen-damaging effect of sodium ethoxide on deplasticized epoxy sections. Journal of Histochemistry and Cytochemistry 1997; 45(1): 143-146.

21) Brorson S-H, Strøm EH. A new immunoelectron microscopy approach for the detection of immunoglobulin and complement deposits in epoxy-embedded renal biopsies. Ultrastructural Pathology 1998; 22: 449-457.

22) Brorson S-H. pH-dependent effect of heat-induced antigen retrieval of epoxy sections for electron microscopy. Micron 2002; 33: 481-482.

23) Tano S, Kashima K, Daa T, Urabe S, Tsuji K, Nakayama I *et al.* An antigen retrieval method using an alkaline solution allows immunoelectron microscopic identification of secretory granules in conventional epoxy-embedded tissue sections. Journal of Histochemistry and Cytochemistry 2003; 51(2): 199-204.

24) Bendayan M, Zollinger M. Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. Journal of Histochemistry and Cytochemistry 1983; 31(1): 101-109.

25) Bettica A, Johnson AB. Ultrastructural immunogold labelling of glial filaments in osmicated and unosmicated epoxy-embedded tissue. Journal of Histochemistry and Cytochemistry 1990; 38(1): 103-109.

26) Stirling JW, Graff PS. Antigen unmasking for immunoelectron microscopy: labelling is improved by treating with sodium ethoxide or sodium metaperiodate, then heating on retrieval medium. Journal of Histochemistry and Cytochemistry 1995; 43(2): 115-123.

27) Stirling JW. Ultrastructural localization of lysozyme in human colon eosinophils using the protein A-gold technique: effects of processing on probe distribution. Journal of Histochemistry and Cytochermistry 1989; 37(5): 709-714.

28) Evers P, Uylings HBM, Suurmeijer AJH. Antigen retrieval in formaldehyde-fixed human brain tissue. Methods: A Companion to Methods in Enzymology 1998; 15: 133-140.

29) Shi S-R, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. Journal of Histochemistry and Cytochemistry 1997; 45(3): 327-343.

30) Saito N, Konishi K, Takeda H, Kato M, Sugiyama T, Asaka M. Antigen retrieval trial for post-embedding immunoelectron microscopy by heating with several unmasking solutions. Journal of Histochemistry and Cytochemistry 2003; 51(8): 989-994.

31) Qu-Hong, Dvorak AM. Ultrastructural localization of osteopontin immunoreactivity in phagolysosomes and secretory granules of cells in human intestine. Histochemical Journal 1997; 29: 801-812.

32) Robinson JM, Vandré DD. Antigen retrieval in cells and tissues: enhancement with sodium dodecyl sulphate. Histochemistry and Cell Biology 2001; 116: 119-130.

33) Brorson S-H. Fixative-dependent increase in immunogold labeling following antigen retrieval on acrylic and epoxy sections. Biotechnic and Histochemistry 1999; 74(5): 248-260.

34) Shi S-R, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. Journal of Histochemistry and Cytochemistry 2001; 49(8): 931-937.

35) Shi S-R, Cote RJ, Young L, Imam A, Taylor CR. Use of pH 9.5 Tris-HCl buffer containing 5% urea for antigen retrieval immunohistochemistry. Biotechnic and Histochemistry 1996; 71(4): 190-196.

36) Pileri SA, Roncador G, Ceccarelli C, Piccioli M, Briskomatis A, Sabattini E *et al.* Antigen retrieval techniques in immunohistochemistry: comparison of different methods. Journal of Pathology 1997; 183: 116-123.

37) Jiao Y, Sun Z, Lee T, Fusco FR, Kimble TD, Meade CA *et al.* A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections. Journal of Neuroscience Methods 1999; 93: 149-162.

38) Boenisch T. Heat-induced antigen retrieval restores electrostatic forces: prolonging the antibody incubation as an alternative. Applied Immunohistochemistry and Molecular Morphology 2002; 10(4): 363-367.

39) Xiao J-C, Adam A, Ruck P, Kaiserling E. A comparison of methods for heat-mediated antigen retrieval for immunoelectron microscopy: demonstration of cytokeratin no.18 in normal and neoplastic hepatocytes. Biotechnic and Histochemistry 1996; 71(6): 278-285.

40) McQauid S, McConnell R, McMahon J, Herron B. Microwave antigen retrieval for immunocytochemistry on formalin-fixed, paraffin-embedded post-mortem CNS tissue. Journal of Pathology 1995; 176: 207-216.

41) Ishida-Yamamoto A, Tanaka H, Nakane H, Takahashi H, Iizuka H. Antigen retrieval of loricrin epitopes at desmosomal areas of cornified cell envelopes: an immunoelectron microscopic analysis. Experimental Dermatology 1999; 8: 402-406.

42) Hoetelmans RWM, van Slooten H-J, Keijzer R, van de Velde CJH, van Dierendonck JH. Comparison of the effects of microwave heating and high pressure cooking for antigen retrieval of human and rat Bcl-2 protein in formaldehyde-fixed, paraffin-embedded sections. Biotechnic and Histochemistry 2002; 77(3): 137-144.

43) Röcken C, Roessner A. An evaluation of antigen retrieval procedures for immunoelectron microscopic classification of amyloid deposits. Journal of Histochemistry and Cytochemistry 1999; 47(11): 1385-1394.

44) Cavanna F, Ruggeri G, Iacobello C, Chieregatti G, Murador E, Albertini A *et al.* Development of a monoclonal antibody against human heart ferritin and its application in an immunoradiometric assay. Clinica Chimica Acta 1983; 134: 347-356.

45) Arosio P, Albertini A. Structure and immunochemistry of isoferritins. Italian Journal of Biochemistry 1984; 33(4): 275A-277A.

46) Arosio P, Cozzi A, Ingrassia R, Levi S, Luzzago A, Ruggeri G *et al.* A mutational analysis of the epitopes of recombinant human H-ferritin. Biochimica *et* Biophysica Acta 1990; 1039: 197-203.

47) Roberts DD, Drysdale JW. Immunoreactivities of human isoferritins. Clinica Chimica Acta 1985; 150: 41-51.

48) Luzzago A, Arosio P, Iacobello C, Ruggeri G, Capucci L, Brocchi E *et al.* Immunochemical characterization of human liver and heart ferritins with monoclonal antibodies. Biochimica *et* Biophysica Acta 1986; 872: 61-71.

49) Hoy TG, Jacobs A. Changes in the characteristics and distribution of ferritin in iron-loaded cell cultures. Biochemical Journal 1981; 193(1): 87-92.

50) Hoy TG, Jacobs A. Ferritin polymers and the formation of haemosiderin. British Journal of Haematology 1981; 49(4): 593-602.

51) Thompson KJ, Fried MG, Ye Z, Boyer P, Connor JR. Regulation, mechanisms and proposed function of ferritin translocation to cell nuclei. Journal of Cell Science 2002; 115: 2165-2177.

52) Surguladze N, Patton S, Cozzi A, Fried MG, Connor JR. Characterization of nuclear ferritin and mechanism of translocation. Biochemical Journal 2005; 388: 731-740.

53) Roth J. The silver anniversary of gold: 25 years of the colloidal gold marker system for immunocytochemistry and histochemistry. Histochemistry and Cell Biology 1996; 106: 1-8.

54) Bendayan M. A review of the potential and versatility of colloidal gold cytochemical labeling for molecular morphology. Biotechnic and Histochemistry 2000; 75(5): 203-242.

55) Brorson S-H. Bovine serum albumin (BSA) as a reagent against non-specific immunogold labelling on LR White and epoxy resin. Micron 1997; 28(3): 189-195.

56) Birrell GB, Hedberg KK, Griffith OH. Pitfalls of immunogold labelling: analysis by light microscopy, transmission electron microscopy, and photoelectron microscopy. Journal of Histochemistry and Cytochemistry 1987; 35(8): 843-853.

57) Kaur R, Dikshit KL, Raje M. Optimization of immunogold labelling TEM: an ELISA-based method for rapid and convenient simulation of processing conditions for quantitative detection of antigen. Journal of Histochemistry and Cytochemistry 2001; 49(3): 355-367.

58) Louw J, Williams K, Harper IS, Walfe-Coote SA. Electron dense artefactual deposits in tissue sections: the role of ethanol, uranyl acetate and phosphate buffer. Stain Technology 1990; 65(5): 243-250.

59) Santambrogio P, Levi S, Cozzi A, Rovida E, Albertini A, Arosio P. Production and characterization of recombinant heteropolymers of human ferritin H and L chains. Journal of Biological Chemistry 1993; 268(7): 12744-12748.