Immunology 1992 77 267–276

Mycobacterial 65,000 MW heat-shock protein shares a carboxy-terminal epitope with human epidermal cytokeratin 1/2

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Accepted for publication 4 May 1992

SUMMARY

Molecular mimicry between mycobacterial heat-shock protein (hsp) 65 and host tissue antigens have been implicated in the autoimmune pathogenesis of certain idiopathic diseases. Here, we demonstrated that two of our previously characterized monoclonal antibodies (mAb), Ne5 and Nd4 that were directed to a carboxy-terminal epitope on the mycobacterial hsp 65, specifically cross-reacted with suprabasal cytokeratin of the normal human skin. These mAb also showed similar keratin staining of hair follicle epithelia and produced no reaction with other dermal components. Both mAb strongly stained the cytoplasm of the majority of freshly isolated epidermal keratinocytes from the normal human skin. None of these mAb showed staining with human HeLa cells and with human skin fibroblasts. Immunoblotting using total keratin extract prepared from isolated epidermal keratinocytes revealed that mAb Ne5 and Nd4 specifically reacted with a molecular size of 65,000–67,000 MW keratin protein(s) and such reactivity was not observed from cytoskeletal proteins extracted from HeLa cells and skin fibroblasts. Comparison of immunoblotting reactivity with conventional anti-cytokeratin mAb further revealed that mAb Ne5/Nd4 recognized a 65,000–67,000 MW molecular-sized protein corresponding to cytokeratin 1/2 from the same keratinocyte extract as anti-cytokeratin mAb. Preincubation of mAb Ne5/Nd4 with the purified mycobacterial hsp 65 abolished this keratin cross-reactivity in both immunohistochemistry and immunoblotting. Moreover, these mAb showed no keratin staining in lesional psoriatic skin and also reacted weakly with cultured epidermal keratinocytes. Since mAb Ne5/Nd4 specifically recognized a 67,000–65,000 MW molecular-sized protein(s) derived from epidermal keratinocytes and the known characteristics of epidermal cytokeratin 1/2 appeared to be consistent with present results, we concluded that Ne5/Nd4 cross-reactive protein(s) in the human epidermis is suprabasal cytokeratin 1/2. Comparison of the previously mapped Ne5/Nd4 epitope region of amino acid residues 525–540 of the mycobacterial hsp 65 with the entire sequence of human 65,000 MW keratin revealed that a stretch of nine amino acids of the Ne5/Nd4 epitope sequence resembled certain regions of the carboxy-terminus of the human 65,000 MW keratin. This similarity of the mycobacterial hsp 65 probably contributes to the cytokeratin cross-reactive epitope. Our results presented here demonstrate direct evidence of immunological cross-reactivity between mycobacterial hsp 65 and human epidermal cytokeratin 1/2. We speculate that Ne5/Nd4 cross-reactive epitope of epidermal cytokeratins might be an important target for skin diseases.

INTRODUCTION

Mycobacteria are in the list of candidate microbes that have been suggested to have an aetiological role in autoimmune diseases.[1–3] On the other hand, the occurrence of autoimmune tissue injuries is quite common in active mycobacterial diseases such as leprosy.[3] Furthermore, antigenic similarity between *Mycobacterium leprae* and human nerve as well as skin components have been suggested as being responsible for the development of autoimmune clinical manifestations of leprosy.[4]

The mycobacterial 65,000 MW is one of the well-characterized immunodominant proteins which share extensive sequence homology with GroEL heat-shock protein (hsp) 65[5] and human mitochondrial hsp 65 or P1 protein.[6] Such sequence homology makes this mycobacterial protein a potential inducer of cross-reactive immune responses to host self molecules that may lead to autoimmunity.[2,7,8] This hypothesis has often been suggested

Abbreviations: BCG, bacillus Calmette–Guérin; C-terminal, carboxy-terminal; hsp, heat-shock protein; mAb, monoclonal antibodies; SDS–PAGE, sodium dodecyl sulphate polyacryl gel electrophoresis; MW, molecular weight; TMB, 3,3′,5,5′-tetramethylbenzidine.

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for the pathogenesis of several diseases without any active mycobacterial infection.[2,7] A well-documented example is the antigenic mimicry between mycobacterial hsp 65 and cartilage proteoglycans that has been implicated for the pathogenesis of autoimmune arthritis in rat and in human.[9-11] In addition, elevated levels of antibodies against mycobacterial hsp 65 in sera of rheumatoid arthritis patients[12] and also the presence of mycobacterial hsp 65 reactive T cells in the synovium have been reported.[13] Moreover, recent studies have also shown that the mycobacterial hsp 65 could be associated with the development of spontaneous diabetes in non-obese diabetic mice.[14]

The aetiology of common inflammatory skin diseases is not known. However, autoimmunity and the involvement of various microbial agents have been strongly suggested as playing a role in the aetiopathogenesis of the disease.[15,16] Moreover, circulating autoantibodies to various skin components including keratin proteins, and the presence of arthritic complications in skin diseases, especially in patients with psoriasis, suggest the existence of autoimmunity in these patients.[15,17,18] Evidence which suggests that mycobacteria may be involved in the pathogenesis of skin diseases is the detection of antibodies of immunodominant mycobacterial antigens in sera from these patients.[19] More interestingly, we recently found that psoriasis patients showed significantly high serum IgG antibody activity to mycobacterial hsp 65 and other immunodominant cross-reactive mycobacterial antigens.[20] In this context, it is important to investigate the possible antigenic mimicry between mycobacterial antigens, particularly mycobacterial hsp 65 and skin components in relation to the cutaneous pathology of inflammatory skin diseases. Therefore, we carried out an immunohistochemical analysis on skin biopsies from various patient groups with special reference to psoriasis, and normal individuals using a novel series of monoclonal antibodies (mAb) against mycobacterial hsp 65. During this investigation, we identified that two of our newly characterized mAb, Ne5 and Nd4,[21] specifically cross-reacted with suprabasal cytokeratins of normal human epidermis. Consequently, we extended this study further to characterize the nature of this cross-reactive epitope.

In this report we documented that a C-terminal epitope of the mycobacterial hsp 65 is shared with human epidermal suprabasal 67,000/65,000 MW cytokeratin or K1/K2. We also documented here that the amino acid sequence of this epitope on mycobacterial hsp 65 shows limited sequence homology with human 65,000 MW cytokeratin, and thus demonstrate a novel example of molecular mimicry between mycobacterial hsp 65 and human tissues.

MATERIALS AND METHODS

Monoclonal antibodies

The two novel mAb designated as Ne5 and Nd4 of IgG1 isotype directed to *Mycobacterium tuberculosis/Mycobacterium bovis* bacillus Calmett–Guérin (BCG) 65,000 MW hsp that were used in this study, have been described previously.[21] Characterization of these two mAb with *M. bovis* BCG 65,000 MW fusion or truncated proteins expressed in *Escherichia coli* encoding various segments of the molecule revealed that these mAb defined epitopes reside in the amino acid residues 525–540.[21] Other anti-mycobacterial mAb that are directed to the same amino acid region but to different epitopes of the mycobacterial hsp 65 used in this study were mAb F67-2[23] (a kind gift from Dr A. H. J. Kolk, Royal Tropical Institute, Amsterdam, The Netherlands), mAb Pe12 and Rb2 (A. Rambukkana *et al.*, submitted for publication). In addition, mAb were partially purified from culture supernatant by ammonium sulphate precipitation and used in all experiments whereas mAb F67-2 was used as ascitic fluid. Monoclonal antibodies Ne5 and Nd4 were further purified by affinity chromatography and labelled with horseradish peroxidase (HRP) as described previously.[21,24]

We also used conventional anti-cytokeratin mAb, 34βB4[25] and RKSE60[26] which are directed against human suprabasal cytokeratin 1/2 and 10/11, and were purchased from Enzo Diagnostic and Organon Teknika, (The Netherlands) respectively.

Tissue specimens

Skin biopsies studied were from patients with psoriasis ($n=7$) (both lesional skin and normal appearing or uninvolved skin), atopic dermatitis ($n=4$), leprosy [both tuberculoid ($n=4$) and baciliferous lepromatous leprosies ($n=3$)] and normal controls including foreskin ($n=9$). Well-classified leprosy biopsies studied were the same as described in our previous report.[27] Biopsies were snap frozen in liquid nitrogen and stored at $-90°$. Cryostat sections, 6 μm thick, were air dried for 20 min and fixed in pure acetone for 10 min at 4°.

Cell culture

Keratinocytes were isolated from normal human foreskins as previously described[28] using the method of Rheinwald and Green.[29] Briefly, dermoepidermal separation was performed using thermolysin, 0·5 mg/ml (Sigma Chemical Co., St Louis, MO) and keratinocytes were released from the epidermis in 0·25% trypsin. Isolated keratinocytes were cultured up to confluency in DMEM containing 10% foetal calf serum, glutamin (2 mM), penicillin/streptomycin (100 U/ml/0·1 mg/ml), hydrocortisone (0·4 mg/ml) and epidermal growth factor [(10 ng/ml) Sigma] on collagen 1 (vitrogen)-coated culture flasks.[28,29] Experiments with cultured keratinocytes were performed on cells with four passages. Purity of keratinocyte preparations were evaluated by immunohistochemical analysis as described below using mAb to total keratinocyte marker CK1 (Dako, Glostrup, Denmark), and positive staining was found in more than 90% of the keratinocytes. All human specimens were obtained from the Departments of Dermatology and Pediatrics, Academic Medisch Centrum (AMC), Amsterdam, The Netherlands.

HeLa cell line (obtained from American Type Culture Collection, ATCC, Rockville, MD) and human fibroblast cell line HSF-7 (a gift from Dr D. J. Chen, Los Alamos National Laboratory, NM)[28] used in this study were cultured as previously reported.[28,30] Cytospin preparations of freshly isolated and cultured keratinocytes and cell lines from HeLa and human skin fibroblasts were made by spining down the cells at 800 *g* in a cytospin centrifuge and were fixed with cold acetone.

Immunohistochemical analysis

A two-stage mAb peroxidase-conjugated rabbit anti-mouse immunoglobulin staining system with 3-amino-9-ethyl-carbazol (AEC) as staining reagent was employed on frozen skin section and cytospin preparations as previously described.[31] Briefly, partially purified and ascitic fluid containing anti-mycobacterial

65,000 MW mAb were used at dilutions of 1:10 and 1:50 respectively whereas anti-cytokeratin mAb (ascitic fluid) RKSE60 and 34βB4 were used at 1:100 and 1:500 dilutions respectively. Negative control sections were either overlaid with phosphate-buffered saline (PBS) (pH 7·4) or with irrelevant mAb with same isotype. Sections were then incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin for 30 min. Staining was achieved by incubation with freshly prepared acetate buffer solution (pH 4·9) which contained AEC, dimethylformamide and hydrogen peroxide. The staining was visually controlled and stopped by washing in acetate buffer and counterstained with haematoxylin in acetate buffer for 1 min and finally sections were mounted.

Antigen preparations

Whole sonicate of *M. tuberculosis* (strain RIVM 7114)[32] and the native purified recombinant 65,000 MW protein of *M. bovis* BCG (MbaA) were prepared as previously reported.[24,32,33] Preparation of crude epidermal keratin proteins from freshly isolated and cultured keratinocytes were performed as reported earlier.[34] Other cytoskeletal keratins and vimentin-rich intermediate filament proteins were also extracted similarly from HeLa cells and human skin fibroblast respectively. Briefly, an equal number of cells from each cell type was washed twice with PBS (pH 7·4) and directly lysed by boiling for 5 min in 1% SDS and 25 mM Tris HCl (pH 7·4). Cell debris was removed by centrifugation and the supernatants were extensively dialysed against PBS (pH 7·4), and protein concentration of each fraction was determined.

SDS-PAGE and immunoblotting

SDS-PAGE was performed in slab gels (12%) in a discontinuous Tris-buffer system as described previously.[21,24] All antigen preparations were incubated with sample buffer at 65° for 20 min. A pre-evaluated concentration of 10 μg of proteins was applied from *M. tuberculosis* sonicate whereas 2 μg was loaded from purified mycobacterial hsp 65. From different cytoskeletal preparations, approximately 20 μg of proteins were applied per slot. Separated proteins were transferred to nitrocellulose papers according to Towbin *et al.*[35] and immunoblotting with mAb was performed as previously described.[21,24] Partially purified and ascitic fluid containing anti-65,000 MW mycobacterial mAb were used at dilutions of 1:500 and 1:1000 respectively whereas anti-cytokeratin mAb were used at dilutions of 1:1000 and 1:2000. The secondary HRP-conjugated affinity-purified goat anti-mouse IgG (Tago, Burlingame, CA) was used at 1:1000 dilution. Colour development was performed with TMB substrate solution with dioctyl sodium sulphosuccinate and hydrogen peroxide as described previously.[24] In each experiment, low M_r markers (BioRad, Richmond, CA) were subjected to blotting after SDS–PAGE.

Inhibition studies

Immunoperoxidase and immunoblotting inhibition tests were performed by preincubating appropriately diluted anti-mycobacterial mAb with equal volumes of excess antigens (approximately 5–10 times more than prescribed by the antibody titre). As a negative control inhibitor, 0·5% bovine serum albumin (BSA) was used. Absorbtion was carried out for 1 hr at 37° followed by overnight incubation at 4°. Absorbed mAb were tested by immunohistochemistry and immunoblotting as compared to unabsorbed mAb. In addition, we also studied the inhibition of direct binding of appropriately diluted HRP-labelled mAb Ne5 to the Western blots of keratinocyte extract by preincubation of the blots with conventional anti-cytokeratin mAb 34βB4.[25]

RESULTS

Reactivity of anti-mycobacterial 65,000 MW mAb with human epidermal cytokeratins in immunohistochemistry

During an investigation of the possible involvement of mycobacterial hsp 65 in the cutaneous pathology of unknown inflammatory skin diseases using a novel series of mAb against mycobacterial hsp 65, we identified that two of our well-characterized mAb, Ne5 and Nd4[21] specifically stained the suprabasal cytokeratin of normal human epidermis. In all normal skin biopsies including human foreskin tested, both mAb Ne5 and Nd4 in immunoperoxidase technique demonstrated intense and uniform staining of suprabasal keratin with completely negative basal cell staining (Fig. 1A). These two mAb also showed suprabasal keratin staining with hair follicle; Fig. 2 illustrates the specific keratin staining of longitudinal and cross-sections of hair shafts of a normal skin. These mAb showed no staining with simple epithelia of the sweat gland and also with other dermal components such as fibroblasts, smooth muscles and nerves (Fig. 2A), indicating their cross-reactivity with a specific type of keratin intermediate filaments. The negative immunohistochemical results with other mAb (F67-2, Rb2 and Pe12), which are directed to different epitopes of the same carboxy-terminal region of mycobacterial hsp 65 (Table 1), further indicate the specific nature of mAb Ne5/Nd4.

Interestingly, both mAb Ne5 and Nd4 showed a very weak staining (or in some cases no staining) with all lesional psoriatic skin tested (see Fig. 7B) whereas suprabasal keratin reactivity was seen in non-lesional or normal-appearing epidermis of psoriasis and also in dermatitis patients. In addition, suprabasal keratin staining could also be seen in both lepromatous and tuberculoid leprosy lesions irrespective of the bacilliary load and granuloma formation. However, the staining pattern was found to be slightly weaker in the latter cases as compared to normal epidermis (data not shown).

Specificity analysis of the suprabasal keratin staining by both mAb Ne5 and Nd4 revealed that this reactivity was greatly inhibited by preincubation of these mAb with either purified mycobacterial hsp 65 or the total sonicate of *M. tuberculosis* (Fig. 1B). Since these mAb only reacted with 65,000 MW protein from total mycobacterial sonicate it is suggested that mycobacterial 65,000 MW protein (hsp 65) was responsible for the cross-reaction.

Since the cross-reactivity pattern of anti-mycobacterial mAb in the skin is strongly with suprabasal keratinocytes which constitute the major part of the epidermis, we isolated keratinocytes from normal human foreskin and cytospin preparations were used in immunoperoxidase staining. As expected, comparable to the reactivity pattern of normal epidermis, mAb Ne5 and Nd4 showed strong cytoplasmic staining with the majority of

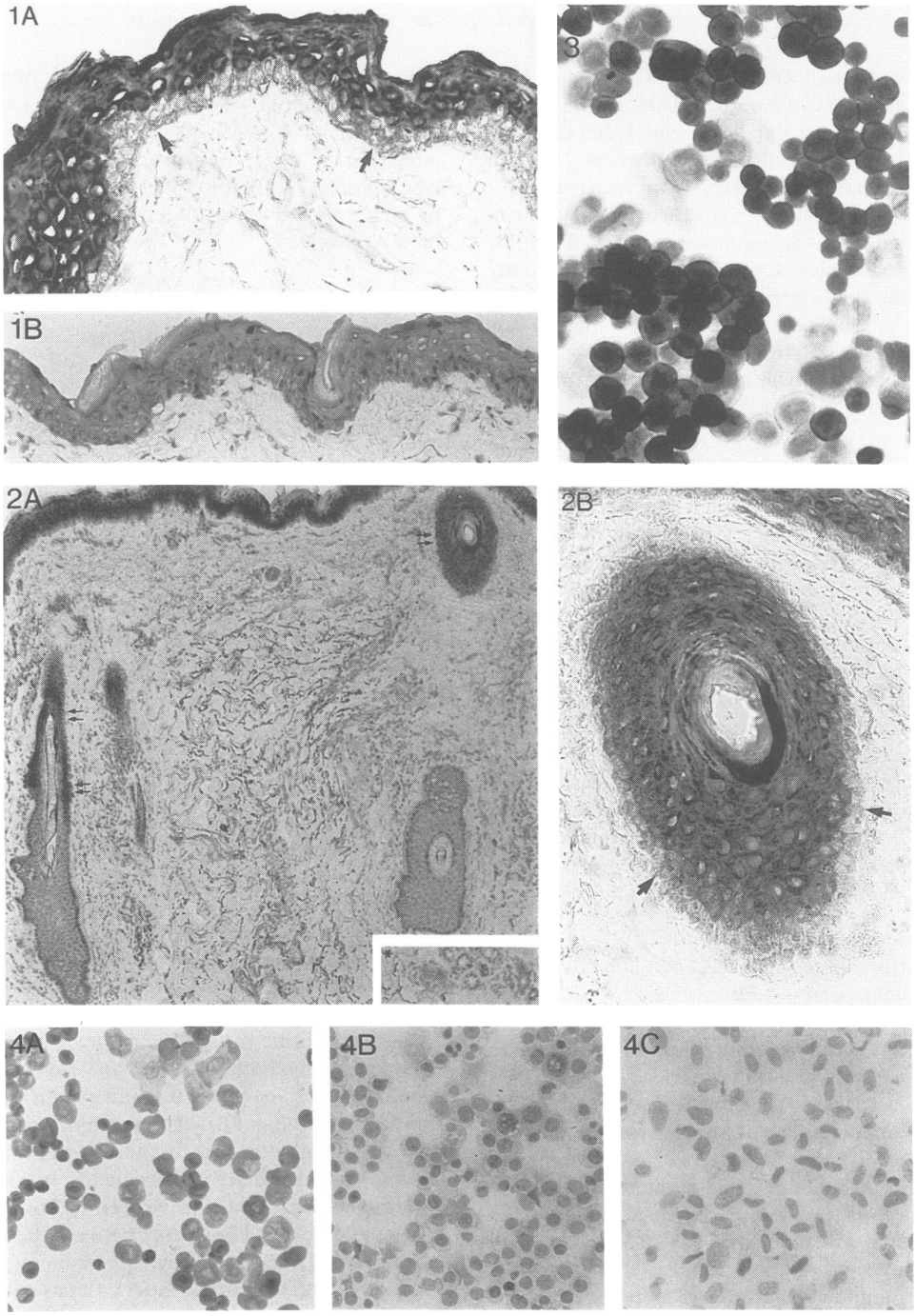

Figure 1. (A) Keratinocytes of the suprabasal layers of normal human epidermis are shown to contain a molecule that is indistinguishable from the highly conserved mycobacterial hsp 65. Immunoperoxidase staining of cryostat section of normal skin with mAb Ne5 against mycobacterial hsp 65. Notice the non-reactivity of basal cells indicated by arrows. (B) Preincubation of mAb Ne5 with mycobacterial hsp 65 caused the disappearance of suprabasal keratin staining. Monoclonal antibody Nd4 showed the similar staining pattern as mAb Ne5. Original magnification: (A) × 700; (B) × 400.

Figure 2. (A) Immunoperoxidase staining of a cryostat of normal skin to show the specific cross-reactivity of mAb Ne5 with suprabasal keratin of the hair follicle epithelium, and the non-reactivity with other dermal components including sweat gland epithelium of the same section.* Note the specific keratin staining of the hair follicle towards the epidermis (double arrows) in both horizontal section and the cross-section. (B) Higher magnification of the same cross-section of hair follicle showing the suprabasal staining (arrows indicated the negative staining of basal layer). Original magnification: (A) × 400; (B) × 2500.

Figure 3. Immunoperoxidase staining of a cytospin preparation of freshly isolated epidermal keratinocytes from human foreskin with mAb Ne5. Original magnification × 2500.

Figure 4. Immunoperoxidase staining of cytospin preparations of (A) cultured keratinocytes, (B) human HeLa cells and (C) human skin fibroblasts with mAb Ne5. Original magnification × 1000.

All sections and cytospins (Figs 1–4) were counterstained with haematoxylin.

Table 1. Characteristics of mAb against different epitopes on the C-terminal end of mycobacterial hsp 65

mAb*	Ig class	Epitope region of mycobacterial hsp 65	Cross-reactivity with epidermal keratin†
Ne5	IgG1	residues 525–540[21]	+++
Nd4	IgG1	residues 525–540[21]	+++
F67.2	IgG	residues 481–493[23]	–
Rb2‡	IgG1	residues 479–540	–
Pe12‡	IgG1	residues 479–540	–

* All these mAb defined epitopes on mycobacterial hsp 65 are present in limited number of mycobacterial species.[21,23] ELISA inhibition experiments revealed that Ne5 and Nd4 reactive epitope is different from the rest of the mAb used in this study.[21]

† Immunoperoxidase staining of epidermal suprabasal keratin in normal skin biopsies (mean values of nine specimens) was graded from no staining (–) to maximum brightness (+++).

‡ Prepared in the Dept. of Dermatology and Pathology, Academisch Medisch Centrum, Amsterdam, The Netherlands (A. Rambukkana *et al.*, submitted for publication).

Table 2. Occurrence of tripeptide identities between a nine amino acid stretch of the Ne5 epitope sequence[21] of *M. tuberculosis*/*M. bovis* BCG hsp 65 (530–538) and the entire sequence of human 65,000 MW keratin protein[37]

Tripeptides of Ne5/Nd4 epitope		Occurrence of homologous tripeptides in 65,000 MW keratin	
Amino acid sequence	Residues	Amino terminal	Carboxy-terminal
Pro Gly Gly	530–532	4	—
Gly Gly Gly	531–532	8	9
Gly Gly Asp	532–534	1	—
Met Gly Gly	535–537	1	1
Gly Gly Met	536–538	1	—

freshly isolated keratinocytes (Fig. 3). The staining of mAb with these freshly isolated keratinocytes was also inhibited by preincubation of mAb with either mycobacterial hsp 65 or total sonicate of *M. tuberculosis*. In contrast, when keratinocytes were cultured up to four passages, mAb showed a weak staining pattern as compared to freshly isolated keratinocytes (Fig. 4A). In addition, both mAb produced no staining in HeLa cells and in human skin fibroblasts (Fig. 4B,C), further indicating the specific cross-reactivity of these mAb with a keratin(s) derived from human keratinocytes.

Identification of cross-reactive keratin protein(s)

Human epidermal suprabasal keratinocytes express two major groups of keratins or cytokeratins, proteins of MW of 67,000/65,000 [keratin 1(K1)/keratin 2(K2)] and 57,000/56,000 [keratin 10 (K10)/keratin 11(K11)].[34] To identify our anti-mycobacterial mAb cross-reactive suprabasal keratin proteins, keratins were extracted from epidermal kertinocytes and analysed by SDS–PAGE, electroblotted and probed with mAb. Keratins extracted by this procedure yielded identical SDS–PAGE patterns of protein bands as reported earlier by other investigators.[17,30,34] There is some controversy regarding the identification of closely related 65,000–67,000 MW keratins, specially with anti-cytokeratin polyclonal and monoclonal antibodies as both of these keratins appear in SDS–PAGE as a single protein band, and it is also considered that 65,000 MW keratin is a proteolitic degradation product of 67,000 MW keratin.[36–38] Among the many keratin protein bands of the Western blots of keratinocyte extract [detected by amido black staining (Fig. 5)], mAb Ne5 and Nd4 reacted specifically with a single protein band corresponding to 67,000/65,000 MW cytokeratin(s). No specific binding was detected in proteins extracted from non-keratinizing HeLa cells. Specific binding of mAb to the 65,000 MW protein from *M. tuberculosis* sonicate and purified mycobacterial hsp 65 (Fig. 5, lanes 3 and 4) and to a similar molecular-sized keratin protein(s) (Fig. 5, lane 1) indicated that these mAb recognized a shared epitope from two diverse proteins originating from the mycobacteria and the human host.

To determine whether mycobacterial hsp 65 could inhibit the cross-reactivity of mAb with epidermal cytokeratin, immunoblotting inhibition test was performed. Binding of mAb to the 67,000/65,000 MW keratinocyte-derived protein(s) was completely inhibited by absorbtion of these mAb with the purified mycobacterial hsp 65 or with whole *M. tuberculosis* sonicate. Furthermore, binding of mAb was also inhibited by preincubation of mAb with SDS-extracted cytoskeletal proteins from freshly isolated keratinocytes but not from HeLa cells or human skin fibroblast (data not shown), indicating that the Ne5/Nd4 epitope resides on both mycobacterial hsp 65 as well as on similar molecular-sized keratin proteins derived from human epidermal keratinocytes.

These results suggested that mAb Ne5/Nd4 cross-reactive protein is 67,000/65,000 MW human epidermal cytokeratin(s) or K1/K2. To confirm this theory, we investigated whether previously reported conventional anti-cytokeratin mAb directed against human suprabasal keratin, K1/K2 and K10/K11, would react with the similar molecular-sized keratin protein(s) extracted from the same epidermal keratinocyte preparation. Moreover, since the differentiation-associated suprabasal keratins, especially 67,000/65,000 MW keratins, are known to be greatly reduced in cultured keratinocytes,[34,39] we also looked for the keratin profiles recognized by anti-cytokeratin mAb as compared to cross-reactive Ne5/Nd4 mAb. Anti-cytokeratin mAb 34βB4[25] reacted strongly in Western blots of freshly isolated keratinocytes with 67,000/65,000 MW and 57,000/56,000 MW keratin whereas mAb RKSE60[26] showed a strong band in 57,000/56,000 MW keratin and a weak reactivity with 67,000/65,000 MW keratin (Fig. 6). The immunoblotting reactivity patterns of these anti-cytokeratin mAb with keratin extract preparations are consistent with published results originally described for these anti-cytokeratin mAb.[25,26] As illustrated in Fig. 6, our mAb Ne5/Nd4 against mycobacterial hsp 65 cross-reacted with a similar molecular-sized 67,000/65,000 MW human keratin protein(s) from freshly isolated keratinocytes in a pattern identical to that of conventional anti-cytokeratin 1/2 mAb. Monoclonal antibodies 34βB4 and RKSE60 as well as cross-reactive mAb Ne5/Nd4 did not react

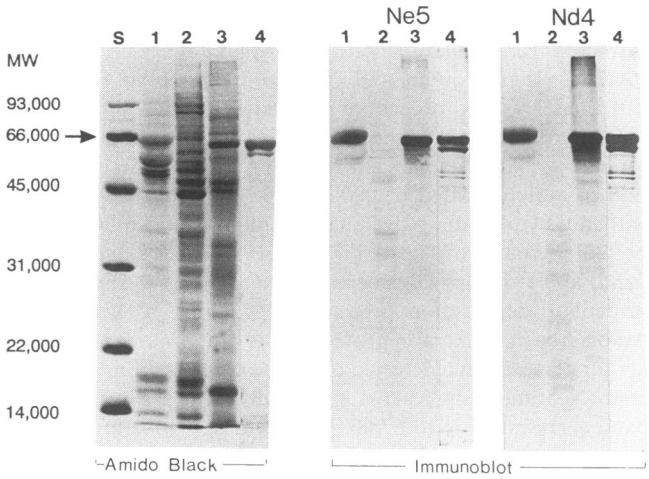

Figure 5. Immunoblotting analysis of mAb Ne5 and Nd4 with epidermal keratins and mycobacterial 65,000 MW protein. Amido black-stained Western blot (left) containing freshly isolated keratinocyte extract (lane 1), HeLa cell extract (lane 2), *M. tuberculosis* whole sonicate (lane 3), purified mycobacterial hsp 65 (lane 4). Immunostaining of the same blots with mAb Ne5 and Nd4 (right). MW markers are shown in lane S. Note the specific reactivity of mAb only with similar molecular-sized proteins derived from human epidermal keratins and mycobacteria.

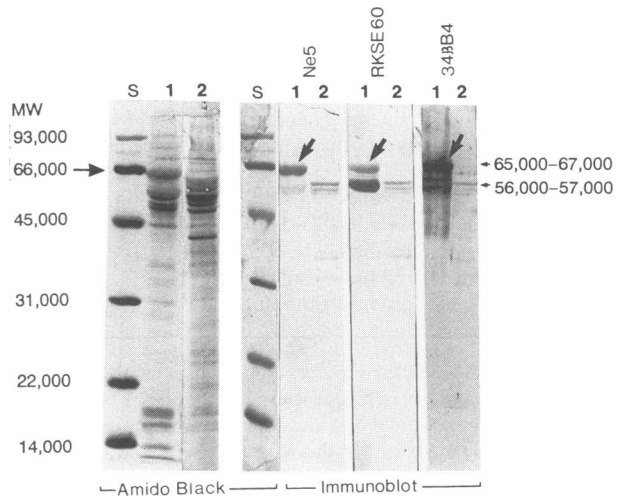

Figure 6. Comparative immunoblot analysis showing the binding of anti-mycobacterial mAb Ne5 and anti-cytokeratin mAb, RKSE60 and 34βB4 to the keratins extracted from freshly isolated (lane 1) and cultured epidermal keratinocytes (lane 2). Amido black-stained Western blots are shown on the left and MW markers are indicated in lane S. Arrows indicate the mAb reactive 67,000/65,000 MW keratin protein(s).

with 67,000/65,000 MW keratin from cultured keratinocyte extract, but all mAb including Ne5/Nd4 showed a similar weak reactivity to a band(s) corresponding to 57,000/56,000 MW keratins. These results further indicated that mAb Ne5 and Nd4 bind to the mycobacterial hsp 65 and to a similar determinant or epitope of human keratin protein(s), providing additional evidence that these mAb cross-reactive protein(s) is epidermal suprabasal 67,000/65,000 MW keratin(s).

To investigate further whether anti-cytokeratin mAb 34βB4, that strongly reacted with 67,000/65,000 MW keratin, recognized the same or a different epitope as cross-reactive mAb Ne5 and Nd4, we performed inhibition immunoblotting assay. The binding of appropriately diluted HRP-labelled mAb Ne5 to the Western blots of freshly isolated keratinocyte extract was not inhibited by preincubation of the blots with excess unlabelled mAb 34βB4 (data not shown). This indicated that mycobacterial cross-reactive Ne5/Nd4 epitope on human 67,000/65,000 MW keratin is different from that of anti-cytokeratin mAb 34βB4 used in this study.

We also compared the epidermal keratin staining of mAb Ne5/Nd4 with that of anti-cytokeratin mAb 34βB4 and RKSE60 with skin biopsies from the normal and other patient groups. Both cross-reactive mAb Ne5 and Nd4 showed similar suprabasal keratin staining as anti-cytokeratin mAb in normal, non-lesional psoriatic skin, atopic dermatitis and leprosy lesions. The major difference of keratin staining between Ne5/Nd4 and anti-cytokeratin mAb was found in the lesional skin from psoriasis patients. As compared to mAb Ne5/Nd4, mAb 34βB4, which reacted mainly to 67,000/65,000 MW keratin but also reacted with 57,000/56,000 MW keratin (Fig. 6)[25] showed a patchy staining in psoriatic lesion (Fig. 7C) whereas RKSE60, which mainly reacted to 57,000/56,000 MW keratin but very weakly reactive to 67,000/65,000 MW keratins (Fig. 6),[26] showed strong keratin staining in the entire suprabasal compartment of psoriatic lesions (Fig. 7D).

Amino acid sequence comparison

Since the keratin cross-reactive mAb Ne5/Nd4 defined epitope on mycobacterial hsp 65 has been previously mapped to a C-terminal sequence of amino acid residues 525–540, we searched for possible homologous amino acid sequence of published human 65,000 MW and 67,000 MW keratin proteins.[37,38] Both these human keratin proteins show 72% sequence homology and run in SDS-PAGE as similar molecular-sized protein.[36–38] The best homology was found between a stretch of nine amino acid residues of the Ne5/Nd4 epitope sequence of *M. tuberculosis*/*M. bovis* BCG hsp 65 with certain regions of the C-terminal end of human 65,000 MW keratin whereas similarities with incomplete C-terminal sequence 67,000 MW keratin[38] were found only with many tripeptide glycine residue (data not shown). The occurrence of identities between the Ne5 epitope and the C-terminal 65,000 MW keratin sequence are shown in Fig. 8. Furthermore, Table 2 shows the overall number of tripeptide identities between the Ne5 epitope sequence and the entire sequence of human 65,000 MW keratin. These similarities at the amino acid level might explain the observed cross-reactivity of mAb Ne5/Nd4 with human epidermal 65,000 MW keratin and indicate that this shared epitope may reside either as a sequential epitope at the C-terminal end or as a conformational epitope on the human 65,000 MW keratin.

In addition, since mycobacterial hsp 65 shows an extensive sequence homology with its human counterpart, the GroEL protein of human hsp 65,[6] we also compared the Ne5/Nd4 epitope sequence with the published sequence of the C-terminal human mitochondrial hsp 65 or P1 protein and its relation to human 65,000 MW keratin. Figure 8 illustrates the identical amino acid residues at the C-terminal end of these three proteins. More interestingly, the C-terminal end of human hsp 65 which partially corresponds to the Ne5/Nd4 epitope sequence, carries characteristic 'keratin-like' Gly Gly Met repeats.[6] The best homology of this region of human hsp 65 included eight identities over a span of nine amino acid residues

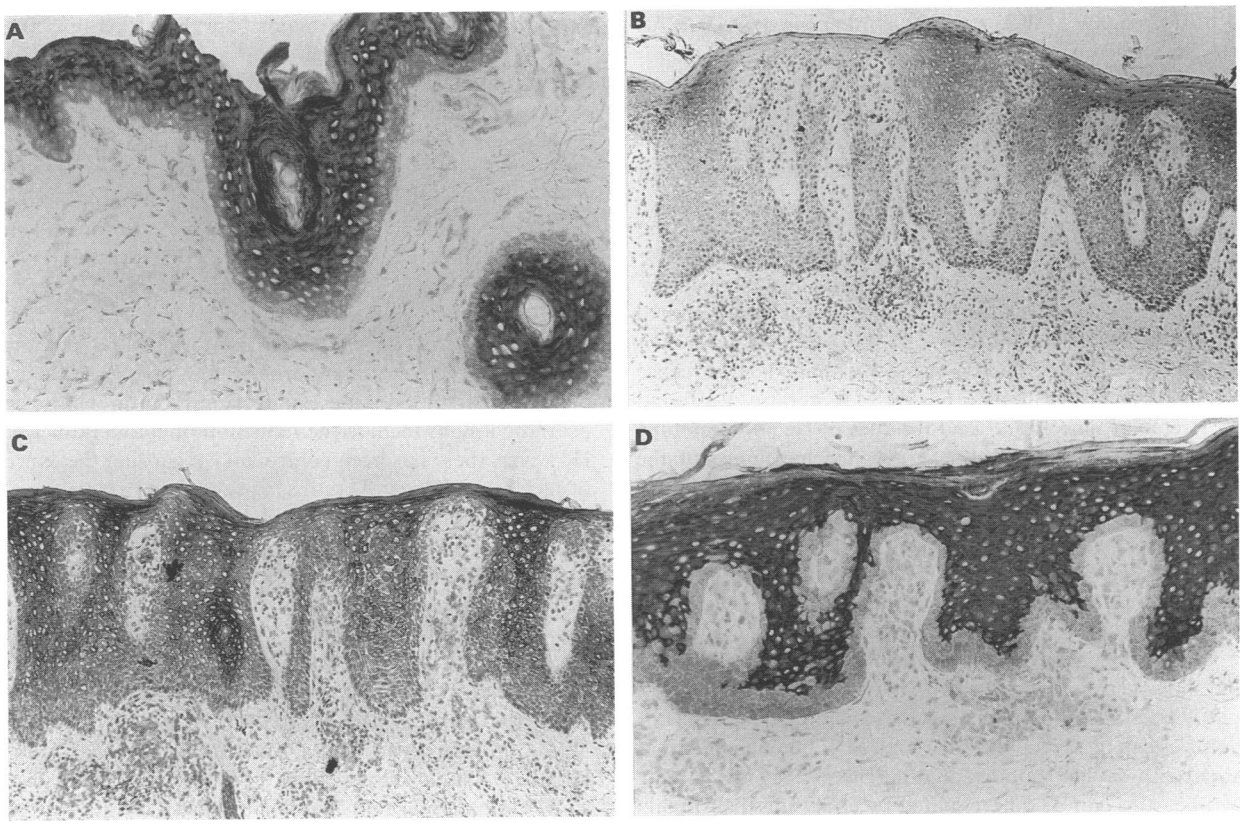

Figure 7. Comparative immunoperoxidase staining of cross-reactive mAb Ne5 and anti-cytokeratin mAb with psoriasis skin. A frozen section of normal-appearing (non-lesional) (A) and lesional psoriatic skin (B) with mAb Ne5. Comparison of the staining pattern of the same lesional psoriatic skin with anti-cytokeratin mAb 34β4 (C) and RKSE 60 (D). The latter two mAb with non-lesional skin show similar staining pattern as mAb Ne5. Original magnification × 400.

M.tuberculosis hsp65 Ne5 epitope	529	Val	Pro	Gly	Gly	Gly	Asp	Met	Gly	Gly	537
Human 65,000 MW keratin	540	Gly*	Gly*	Tyr	Gly*	Gly*	Gly*	Met*	Gly*	Gly*	548
	571	Gly	Ile	Gly	Gly	Gly	Phe	Gly	Gly	Gly	579
	599	Val	Ser	Gly	Gly	Gly	Phe	Ser	Ser	Ala	607
Human hsp65 (P1)	562	Gly*	Gly*	Met	Gly*	Gly*	Gly*	Met*	Gly*	Gly*	570

Carboxy-terminal amino acid sequences

Figure 8. Comparison of C-terminal ends of *M. tuberculosis* hsp 65 (mAb Ne5/Nd4 epitope-containing region)[21] with human 65,000 MW keratin[37] and human hsp 65 or P1 protein[6]. Amino acid sequences are shown using three amino acid letter codes and the residues common to Ne5/Nd4 sequence are boxed. Residues with asterisks indicate the identical amino acids (8/9) between human 65,000 MW keratin (residues 540–548) and human hsp 65.

and has an uninterrupted 6-residue identity with the sequence of 540–548 of the human 65,000 MW keratin.

DISCUSSION

The study described herein demonstrated that two newly prepared mAb raised against a carboxy-terminal epitope on *M. tuberculosis*/*M. bovis* BCG hsp 65 specifically cross-reacted with a suprabasal keratin of the normal human skin. We also show that these mAb strongly stained the cytoplasm of freshly isolated epidermal keratinocytes. By immunoblotting using keratin extract prepared from these epidermal keratinocytes, we identified 67,000/65,000 MW protein(s) as the only mAb cross-reactive keratin(s) that is responsible for the immunohistochemical staining of the suprabasal keratin of the normal human epidermis. Comparing the immunoblotting reactivity with a known anti-cytokeratin mAb 34βB4,[25] it was evident that Ne5/Nd4 cross-reactive mAb also recognized the same molecular-

sized 67,000/65,000 MW keratin(s) (cytokeratin 1/2), as mAb 34βB4. Since the known characteristics of epidermal cytokeratin 1/2 (both biochemical and *in situ* expression pattern) appeared to be consistent with present data obtained from mAb Ne5/Nd4, we provide evidence of immunological cross-reactivity between the mycobacterial hsp 65 and human epidermal cytokeratin 1/2.

The basis for the first phase of molecular mimicry is the sharing of a linear or of conformational fit between a microbe and a host self determinant.[1] We have previously mapped the cytokeratin cross-reactive mAb Ne5/Nd4 to a carboxy-terminal epitope of the amino acid residues 525–540 of the *M. tuberculosis*/*M. bovis* BCG hsp 65.[29] Comparing this amino acid sequence with that of published human 65,000 MW keratin, we found that a stretch of nine amino acid residues of Ne5/Nd4 epitope sequence resembles the variable non-α-helicle regions of the human 65,000 MW keratin. In the carboxy-terminal region of 65,000 MW keratin, there were three different nine amino acid sequences which showed the best homologies. In two of these sequences of 65,000 MW keratin, five of the nine residues were identical whereas in the other, four of the nine were similar to the Ne5/Nd4 sequence. Interestingly, this nine amino acid stretch of Ne5/Nd4 epitope is the only sequence of the mycobacterial hsp 65 that shows certain homology with human 65,000 MW keratin (Fig. 8).[33,37] On the other hand, significant amino acid sequence homologies can be seen in different high MW human epidermal keratins with several important differences.[36–38] Such closely related keratins can be differentiated by analysing their highly variable last 20 amino acid residues in the C-terminal subdomains.[36–38] Many of the previously characterized anti-cytokeratin polyclonal and monoclonal antibodies (especially to 56,000–65,000/67,000 MW keratins) cross-react with more than one related keratin proteins.[25,35,36] The observed specific cross-reactivity of mAb Ne5/Nd4 only with 67,000/65,000 MW keratins probably due to the recognition of such critical amino acid residues in the C-terminal region of 67,000/65,000 MW keratins. In addition, the non-reactivity of these mAb with other intermediate filament proteins, such as vimentin (although vimentin and K1/K2 have diverged from a common ancestral gene)[37] also suggest that this cross-reactive epitope on 67,000/65,000 MW keratin(s) should be present in the hypervariable C-terminal region of the molecule, because the α-helicle regions of the 67,000/65,000 MW keratins are highly conserved among all intermediate filament proteins.[37,38] On the other hand, five different overlapping tripeptides of this particular nine amino acid sequence of Ne5/Nd4 epitope showed identities in 25 different occasions in both N-terminal and C-terminal hypervariable regions of the human 65,000 MW keratin. These similarities may provide evidence that the Ne5/Nd4 epitope could be a conformational rather than a sequential determinant on human 65,000 MW keratin. Since previous studies have shown that the cross-reactive determinants of microbes and host antigens can be limited to as few as four to five amino acid residues,[1,2] it is possible that the cross-reactivity of mAb Ne5/Nd4 with human epidermal cytokeratin is due to such amino acid similarities.

Human keratins are a family of proteins of 40,000–67,000 MW which assemble into 8-nm structural intermediate filaments in the cytoplasm of all epidermal cells.[34,36] Some keratins are restricted to the basal cell layers, and others occur exclusively in the suprabasal compartment.[34] The 67,000/65,000 MW keratins (K1/K2) are the major suprabasal keratins that are associated specifically with keratinization and the terminal differentiation.[34,36–38] One function of this particular keratin(s) is to provide, together with the other components of stratum corneum, an impermeable mechanically resistant shield that forms the first barrier between host and pathogens. It is striking that an immunodominant protein of pathogenic mycobacteria (65,000 MW) shared certain epitopes (Ne5/Nd4) with keratinization-specific epidermal keratins (K1/K2). Interestingly this Ne5/Nd4 epitope is very weakly expressed in the epidermis of all lesional psoriasis biopsies tested. It is known that keratins show characteristic *in situ* expression is normal and pathological epidermis.[34,36,39] Keratin abnormalities in psoriasis skin are well documented.[36,39] Striking changes in keratin expression in psoriatic lesions include the reduction of cytokeratin 1/2.[30,34,39] However, there has been controversy regarding the expression of such high MW cytokeratins in psoriasis;[36,39] this is considered to be due to the fact that the antibodies used for keratin staining of the lesions are directed to the various segments of the carboxy-terminal amino acid residues of high MW cytokeratins.[36,37,39] Since the Ne5/Nd4 epitope sequence seems to correspond to certain C-terminal residues of 65,000 MW keratin, it is possible that this particular cross-reactive epitope on 65,000 MW keratin (or 67,000 MW keratin) is absent or weakly expressed in psoriatic lesion. In contrast, anti-cytokeratin mAb 34βB4, mainly directed to an epitope on 67,000/65,000 MW keratin(s) but also present in 57,000/56,000 MW keratins,[25] showed a patchy keratin staining in psoriatic lesions. In normal skin, suprabasal keratin staining by mAb 34βB4 and Ne5/Nd4 is indistinguishable (Fig. 1)[25] but they differ in their expression in psoriatic lesions (Fig. 7B,C). On the other hand, such difference of keratin staining in psoriatic lesions may be due to the non-reactivity of mAb Ne5/Nd4 with 57,000/56,000 MW keratins (Figs 5 and 6), and the reactivity of mAb 34βB4 with 57,000/56,000 MW keratin (Fig. 6)[25] which are known to express exclusively in psoriatic lesions.[39] This was evident by the strong keratin staining of mAb RKSE60 (Fig. 7C) which mainly recognized the 57,000/56,000 MW keratins.[26] These comparative data further substantiate the evidence of specific immunological cross-reactivity of mAb Ne5/Nd4 with cytokeratin 1/2.

Previous studies have shown that the mycobacterial hsp 65 features epitopes that are shared with several autoantigens, including intracellular components from human.[10,13,14,40] The present finding of immunological cross-reactivity between mycobacterial hsp 65 and epidermal cytokeratin 1/2 adds a novel example to the list of shared epitopes of mycobacterial hsp 65 with human host tissue components. However, the role of immunological cross-reactivity between mycobacterial hsp 65 and human keratin 1/2 in relation to the disease pathogenesis remain unknown. It has been suggested that mycobacteria or microbes in general can induce autoimmune responses through shared determinants on molecules normally present on host cells, by altering the host immune system.[1,2,7,8] Such molecular mimicry can lead to autoimmunity when a host immune response raised against a sequence from a microbe cross-reacts with a host self sequence.[1,2] Interestingly, the existence of mycobacteria-induced immune response in psoriasis patients who showed abnormalities of cytokeratin 1/2, was evident from our recent finding of elevated antibody responses to mycobacterial hsp 65 and other immunodominant mycobacterial antigens.[20] In this context, it is of particular interest whether high

levels of anti-mycobacterial hsp 65 antibody titre as well as reported autoantibodies to various cytokeratins including cytokeratin 1/2 seen in psoriasis patients[17,20] have any relation to the observed cross-reactivity between mycobacterial hsp 65 and human cytokeratin 1/2.

ACKNOWLEDGMENTS

This work was carried out under project no. GNK/DA/004 (AHG-49) of the Faculty of Medicine, University of Amsterdam, The Netherlands. We are indebted to Gastmann-Wichers-Stichting for part financial assistance during this investigation. We are grateful to Dr. J. D. A. van Embden of National Institute of Public Health, Bilthovan, The Netherlands, for providing the *E. coli*-derived *M. bovis* BCG hsp 65 and to the staff of the photographic dept. in AMC for illustrations. Part of this study was presented at the 1st International Conference of the pathogenesis of Mycobacterial infections, 1990, Stockholm, Sweden, and the 20th annual meeting of the European Society for Dermatological Research (ESDR), 1990, Turin, Italy.[22]

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