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Monoclonal Autoantibodies from Patients with Autoimmune Diseases: Specificity, Affinity and Crossreactivity of MAbs Binding to Cytoskeletal and Nucleolar Epitopes, Cartilage Antigens and Mycobacterial Heat-Shock Protein 60

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Abstract

Serum autoantibodies produce typical immunofluorescence staining patterns on HEp-2 cells, which are frequently used for diagnostic purposes. These include antibodies recognizing cytoskeletal and nuclear epitopes. The detailed analysis of human monoclonal antibodies (MAbs) should help to understand which antigens or autoantigens were involved in the generation of these immune responses. Here, three MAbs are described staining HEp-2 cells in a characteristic pattern. They were derived from peripheral blood B cells of two patients with rheumatic diseases (rheumatoid arthritis and relapsing polychondritis). Their binding reactivities were characterized in detail in several assay systems and their affinities measured. Although the antibodies differed in their fine specificity and crossreactivity, all three MAbs (2 IgM, 1 IgA) bound to purified cytoskeletal antigens (desmin) and, in addition, to cartilage antigens (human collagen type II, proteoglycans). The binding to HEp-2 cells could be inhibited specifically with soluble antigens as shown by intracellular flow cytometry. The affinities for both groups of antigens were relatively high (examples: K_D (desmin) = 0.1×10^{-7} M; K_D (collagen) = 3.5×10^{-7} M). Two of the MAbs also bound to heat-shock protein 60 (HSP60) derived from *Mycobacterium tuberculosis.* The results prove that antibodies and B cells with reactivity to both intracellular cytoskeletal and nuclear antigens and exogenous antigens (e. g. HSP60) exist in patients with rheumatic diseases. Similar to an animal model such human B cells may be induced by the exogenous antigen (HSP60) and crossreact with local auto-antigens related to the disease (cartilage). In this way they might contribute to pathogenic processes. Due to their additional crossreactivity with intracellular cytoskeletal and nuclear antigens, these antibodies simultaneously can be detected in the HEp-2 immunofluorescence assay.

Abbreviations: ANA = antinuclear antibodies; HSP60 = heat-shock protein 60; IMF = intermediate filaments; MAb = monoclonal antibody; NAA = natural autoantibodies; MFI = mean fluorescence intensity; RA = rheumatoid arthritis; rPC = relapsing polychondritis.

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Introduction

The screening for antinuclear antibodies (ANA) and antibodies binding to cytoplasmic and cytoskeletal proteins is often performed on HEp-2 cells, a line originated from a human laryngeal carcinoma (epithelioma type 2), grown as monolayers on microscope slides (1). The over 30 different nuclear and cytoplasmic patterns known to occur on HEp-2 cells are produced by many different autoantibodies all contributing to the specificity of the analyzed sera.

Autoantibodies to cytoskeleton proteins are present in a wide range of diseases, including several connective tissue diseases such as rheumatoid arthritis (RA), SLE or systemic sclerosis (2–8). However, they are also present in healthy blood donors, and are then regarded as "natural autoantibodies" (NAA), crossreacting with many bacterial and viral antigens (9–11). Recently, the development of autoantibodies to intermediate filaments (IMF), both keratins and vimentin, was demonstrated in sera of rats, injected with heat killed *Mycobacterium butyricum* in mineral oil, which developed adjuvants arthritis (12).

In many cases it is difficult to understand the relationship between typical staining patterns of autoantibodies and the disease they are correlated with, especially if intracellular antigens are involved (13, 14). It seems unlikely that these antigens themselves are the antigens originally activating the antibody producing B cell. However, to detect the "true" disease related activating antigen – and possible pathogenic mechanisms hidden behind it – may be difficult employing polyclonal body fluids, such as sera. We therefore started to produce and characterize in detail human monoclonal autoantibodies (MAbs) generated from patients with systemic autoimmune diseases (15, 16).

The cytoskeleton consists of three types of filaments – the microfilaments, microtubules and intermediate filaments (IMF) – which differ in diameter and composition (17, 18). Microfilaments contain mainly actin, microtubules tubulin and IMF keratins (type I and II IMF), vimentin, desmin (type III IMF) or other members of the IMF superfamily (type IV, V). IMF are expressed differentially in different cell types and developmental stages, e.g. desmin is synthesized in muscle cells. Type V IMF proteins are the only IMF proteins known to occur in the nucleus where they compose the nuclear lamina.

The aim of the present study was to characterize the binding specificities and affinities of patient derived human MAbs specific for cytoskeleton proteins and for disease related local autoantigens. Here we describe 3 MAbs binding to intracellular antigens as detected on HEp-2 cells. These hybridomas were derived from peripheral blood B cells of two patients, one with rheumatoid arthritis (RA), the other with relapsing polychondritis (rPC) – both diseases with involvement of cartilage.

Materials and Methods

Patients

Patient M (59 years old male) suffered since 17 years from rPC, diagnosed according to the McAdam criteria (19) and by nasal septum biopsy. Additionally he had diabetes mellitus. Patient RA (70 years old male) suffered since 5 years from RA, diagnosed according to the ACR criteria (20). Synovial tissue was obtained at the time of clinically indicated surgery from another patient with RA (70 years old female). Synovial adherent cells were prepared as described (21). Chondrocytes were prepared as described (22) from non-rheumatic autopsy samples of knee cartilage and used before the first passage.

B cell hybridomas

Peripheral blood mononuclear cells (PB-MNC) were T cell depleted by rosetting with sheep erythrocytes and PB B cells transformed with Epstein Barr Virus (EBV) according to standard protocols. From patient RA, two lines were generated from 1×10^6 cells. In the case of patient M, the cells were incubated with the virus for 2 h and then distributed into 3×96 microtiter wells (NUNC, Denmark) containing 10.000, 5.000 or 1.000 cells/well. All cultures were grown up into lines, but 25 were subsequently lost. One line per patient was chosen and used for cell fusion. Briefly, B cells and the heteromyeloma cells CB-F7 (23) (kindly provided by S. Jahn, Berlin, Germany) were washed twice in serum-free culture medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 0.5% penicillin/streptomycin; GIBCO) and mixed in a 5:1 ratio. PEG1500 (Boehringer, Mannheim, Germany) was added (2 µl/10^5 lymphocytes) and stepwise diluted with warm PBS. Cells were centrifuged, resuspended in culture medium (including 10% FCS) and distributed into microtiter plates (4×10^4/culture in 150 µl medium). The next day 50 µl of 4-fold concentrated HAT medium was added. Hybridoma lines were subcloned several times until stable clones were established. The clones M-O and M-P were derived from the same EBV-transformed B cell line (I-A6), but from different hybridoma lines.

Indirect immunofluorescence and flow cytometry analysis

HEp-2 cells and 3T3 cells were obtained from ATCC (Rockville, Maryland, USA). For microscopic analysis cells were grown on glass slides for 12–24 h, washed with serum-free culture medium, air dried and fixed in 100% methanol for 10 min at –20°C. They were then incubated with the first antibodies for 30 min at 20°C in a moist chamber, washed twice in PBS, then again incubated with the second antibodies (FITC-labelled), washed and examined with a fluorescence microscope (Axioscope, Zeiss, Germany). Antibodies were diluted in PBS, 2% FCS, 0.1% NaN_3. In addition, commercially available HEp-2 cell preps were used (Hiss, Freiburg, Germany).

For intracellular flow cytometry, cells were washed in PBS, 3% FCS, 0.1% NaN_3. They were then placed in round bottom 96-well plates (5×10^5 cells/well; NUNC), pelleted and resuspended in ice cold PBS containing 1% paraformaldehyde and 0.25% saponin (Sigma). After 15 min. on ice, cells were washed twice in PBS containing 0.1% saponin and 3% FCS (saponin solution), at 4°C. The pellet was resuspended in antibody- containing saponin solution and incubated 30 min. on ice, followed by washing and incubation with FITC-labelled second reagents. Cells were analyzed by FACScan applying the Lysis II software program (BectonDickinson, Mountain View, CA, USA).

Antigens and antibodies

The following antigens were used: chicken desmin, bovine vimentin, bovine keratin K8, rat actin, hen egg lysozyme (all Boehringer, Mannheim, Germany), keyhole limpet hemocyanin (KLH, Calbiochem, USA), bovine gelatine (Merck, Darmstadt, Germany), human IgG-Fc-fragments (Dianova, Hamburg, Germany). Human collagens Type I, II and V, a human cartilage extract (containing proteoglycans, devoid of collagens) and *Mycobacterium tuberculosis (M. tub.)* HSP60 were prepared as described (24–26) and kindly provided by K. VON DER MARK, Erlangen, and S. KAUFMANN, Berlin, respectively.

Isotype control antibodies (myeloma IgM, pooled serum IgA) were bought from Dianova (Germany). K8.13, V9 (Sigma, Deisenhofen, Germany) and DE-B-5 (Calbiochem) are murine IgG MAbs specific for keratin K8, vimentin and desmin, respectively. Secondary reagents were species-specific goat-anti-Ig antibodies, labelled with FITC for indirect immunofluorescence or with alkaline phosphatase (AP) for ELISAs.

ELISA

ELISAs were performed as described (27). Plates (NUNC) were coated with antigens (usually 5 µg/ml), and blocked with gelatine (0.01%), when appropriate. For quantitation of Ig contents, anti-Ig reagents and isotype controls (Dianova) were used. As detection system we used AP, with para-nitrophenyl phosphate as substrate and read optical densities (OD) at λ = 405 nm with an ELISA reader (EAR 400 AT, SLT, Austria).

Inhibition assays and affinity measurement

Experiments were performed according to the protocol of FRIGUET et al. (28). After determination of useful antibody concentrations, the antibodies were incubated 15 h at room temperature with different concentrations of soluble antigen (up to 6×10^{-7} M). Unbound antibody subsequently was detected on ELISA plates coated with the same antigen. Dissociation constants K_D were calculated from the OD values obtained in the absence and presence of the inhibitor as described (28).

Preparation of protein extracts and immunoblots

Protein extracts were prepared from 1×10^7 HEp-2 cells, washed twice with ice cold PBS and incubated for 30 min on ice with 100 µl lysis buffer (150 mM NaCl, 50 mM Tris pH 8, 1% NP40, protease inhibitors [Complete]; Boehringer, Mannheim, Germany). The supernatant after centrifugation (15 min. 20000 g) was used as extract 1. The pellet was resuspended in the same volume of lysis buffer plus 1.33 M KCl, incubated on ice for 45 min and sonified (10 cycles at 50% power; Sonoplus HD 70, Bandeln, Germany). Then 0.1% SDS was added, the mixture was heated to 95°C for 1 min., and centrifuged as before. The supernatant was used as extract 2. The pellet was resuspended in Laemmli buffer and used as extract 3. Immunoblots were prepared as described (21), using 10% SDS-PAGE, nitrocellulose membranes (Hybond ECL nitrocellulose; Amersham-Buchler, Braunschweig, Germany), blocking with PBS + 5% milk powder + 0.1% Tween 20. The antibodies were added at 5 µg/ml. As secondary reagents goat-anti-human-Ig labelled with HRPO, and as substrate Super signal (Pierce, Rockford, IL, USA) were used.

Results

Generation of human B cell hybridomas

PB B cells derived from a patient with RA (patient RA) were transformed with EBV, and two B cell lines generated, one of which secreted mainly IgA, stained HEp-2 cells (dilution 1:5) and was used for cell fusion. PB B cells from a patient with rPC (patient M) were purified and 263 B cell lines generated by EBV transformation. Supernatants of 5 lines each were mixed in equal volumes (dilution 1:5) and screened on HEp-2 cells with anti-hIgM^{FITC}, -hIgA^{FITC} and -hIgG^{FITC}. All pools contained IgM staining HEp-2 cells, with a large variety of cytoplasmatic and nuclear patterns, not including homogeneous staining of nuclei. IgG and IgA were only detected in 3 pools, staining nuclei (dots), or cytoplasm. One line (M-I-A6) was chosen and used for cell fusion (from 1×10^4 B cells seeded). From both EBV transformed B cell lines several hybridomas were generated and stable monoclonal sublines established. We here present data for 2 MAbs from patient M (M-O and M-P, both IgMλ) and 1 MAb from patient RA (RAB-A, IgA1κ).

Staining patterns of M-O, RAB-A and M-P on adherent cells detected by indirect immunofluorescence

Both original B cell lines produced antibodies which stained the cytoskeleton of HEp-2 cells in a similar way, as shown for the line M-I-A6 in Figure 1a. These patterns resulted from a mixture of antibodies, as both lines were derived from ≥10000 cells originally seeded, and they were later shown to contain at least 5 different clones (by isoelectric focussing and clonal analyses, data not shown). After hybridoma clones were established, we observed that M-O as well as RAB-A bound to 2 different cellular structures, one of

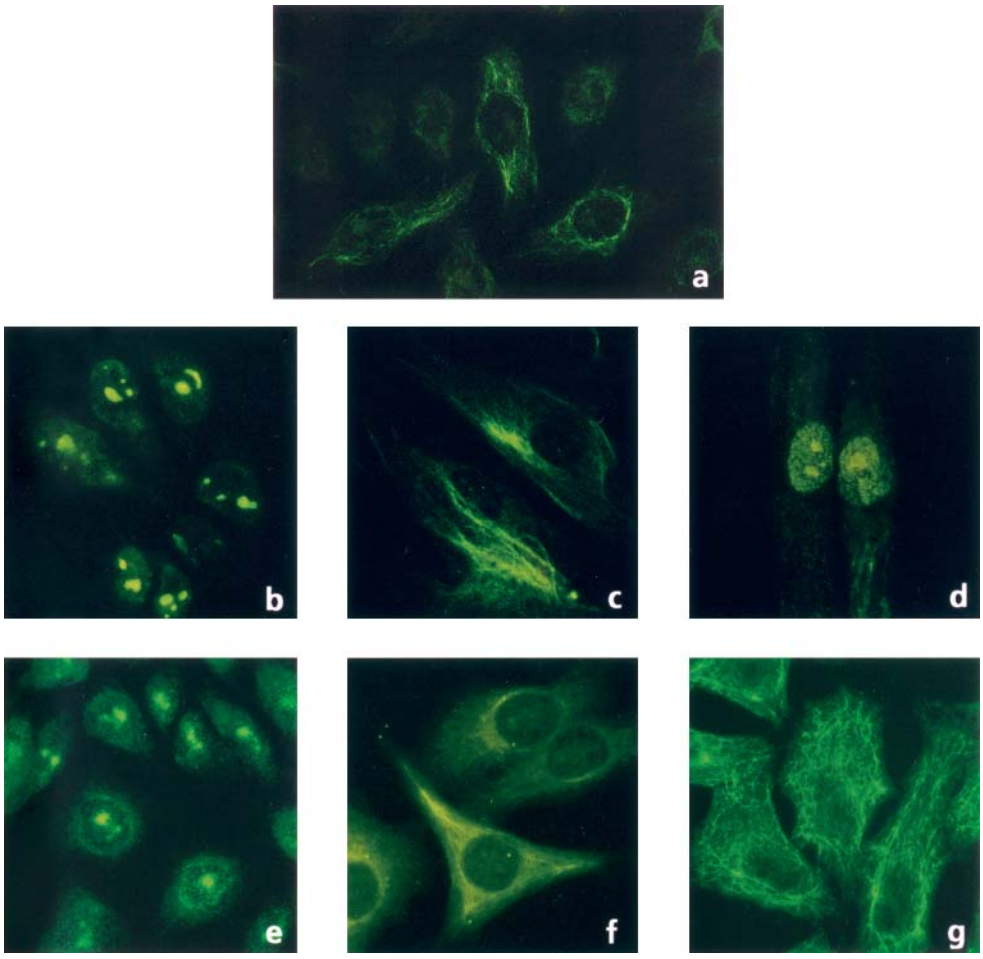


Figure 1. Staining patterns of human monoclonal antibodies (indirect immunofluorescence). **a:** Supernatant of the EBV transformed B cell line M-I-A6 on HEp-2 cells, anti-IgM^{FITC}. **b–d:** M-O on HEp-2 cells (b) and on synovial adherent cells from a RA patient (c, d), anti-IgM^{FITC}, nucleolar (b), cytoplasmatic (c) or nuclear (d) staining. **e, f:** RAB-A on HEp-2 cells, anti-IgA^{FITC}, nucleolar (e) or cytoskeletal (f) staining. **g:** M-P on HEp-2 cells, anti-IgM^{FITC}, cytoskeletal staining. HEp-2 cells were either prepared as described in Materials and Methods (a, b and e) or bought (f, g). Original magnification × 400.

these apparently was a cytoskeletal molecule (Fig. 1c, 1f), whereas the other one was located within the nucleus and/or nucleolus (Fig. 1b, 1d, 1e). The cytoskeletal staining closely resembled the anti-vimentin pattern (1). The clone M-P only bound to cytoskeletal epitopes (Fig. 1g) but not to the nucleus. All three antibodies stained epithelial cells (HEp-2 cells) and mesenchymal cells (murine 3T3 cells, human skin fibroblasts, synovial adherent cells and chondrocytes) with similar patterns (Fig. 1c, 1d). Both patterns

were usually present in the same cell, however, they could also be seen separate from each other, i.e. in different cells of the same preparation (Fig. 1c, 1d). In general, the staining of human cells was stronger than that of rodent cells; the nucleolar pattern seen with M-O was stronger than that seen with RAB-A. Commercially available HEp-2 cells preferentially showed the cytoskeletal pattern, whereas cells prepared in our own laboratory showed both, the cytoskeletal and nuclear pattern.

Quantitative analysis of intracellular epitope binding by cytometry

In addition to the microscopic analyses, the monoclonal antibodies were tested for binding by FACS analyses. Neither M-O nor M-P or RAB-A bound to the cell surface of HEp-2 cells or of PB-MNC (not shown). In contrast, the binding to intracellular epitopes could be quantitatively analysed by flow cytometry. As an example, a histogram of the fluorescence intensities of M-O binding to fixed and permeabilized HEp-2 cells is depicted in Figure 2a, in comparison to an isotype control antibody. Figure 2b illustrates the correlation between the mean fluorescence intensities (MFI) of binding and the concentration of the monoclonal antibodies.

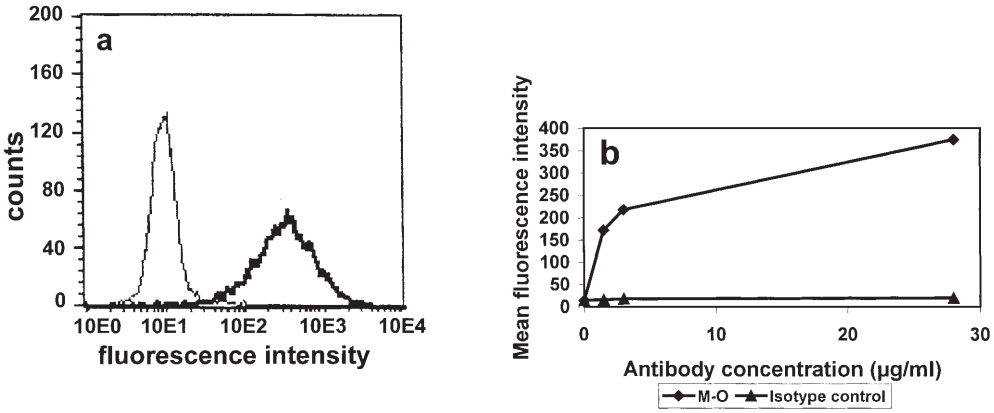


Figure 2. Binding to intracellular epitopes analyzed by cytometry. HEp-2 cells were fixed, permeabilized and then incubated with M-O (dark line) or an isotype control antibody (light line) at a concentration of 28 µg/ml, followed by goat anti-human-IgM^{FITC} (a). The mean fluorescence intensities (MFI) were 375 ± 310.0 (M-O) and 10 ± 4.5 (control), respectively. In addition, titration curves are shown (b).

Antigen binding specificity of M-O, RAB-A and M-P analyzed by ELISA

To characterize the specific binding profiles of the monoclonal antibodies, they were tested at a fixed concentration for binding to a number of cytoskeletal and control antigens in an ELISA. The most interesting results are depicted in Figure 3. M-O was rather specific, binding only to desmin, and not to other cytoskeletal antigens tested like vimentin, keratin K8 or actin. Neither did it bind to a variety of other antigens tested, including

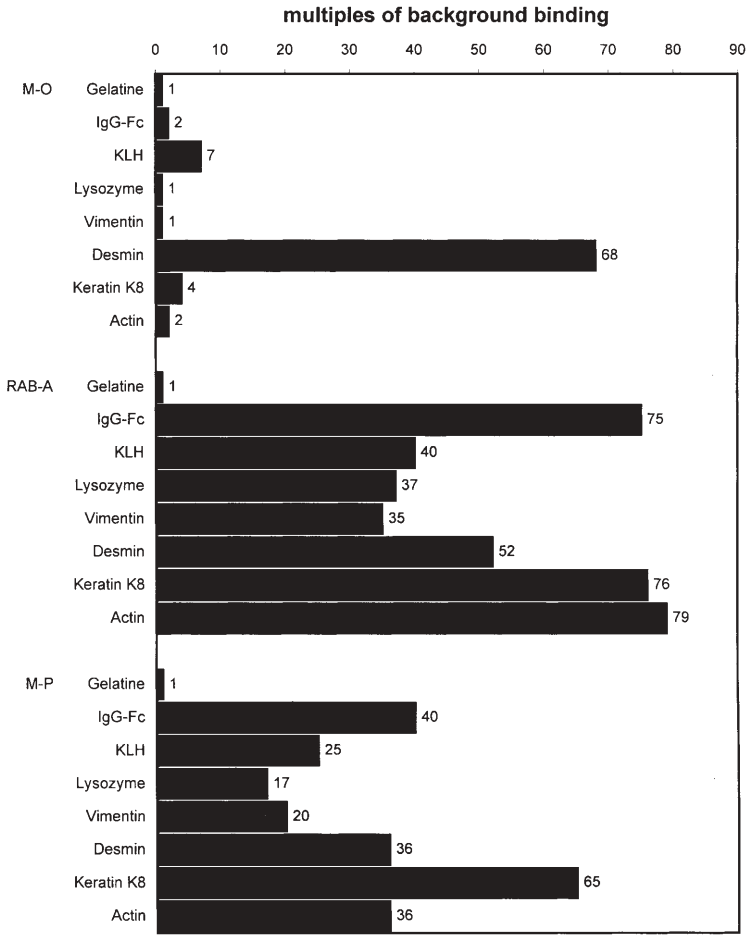


Figure 3. Antigen binding profiles of three MAbs. Antibody containing hybridoma supernatants were standardized to a concentration of 6 μg/ml and incubated on ELISA plates coated with the antigens. Binding was monitored with anti-IgM^{AP} or anti-IgA^{AP}, respectively.

insulin, collagens, albumins, transferrin, lactoferrin or pepsin. In contrast, RAB-A and M-P were polyreactive, binding to all cytoskeletal antigens tested, and in addition to IgG-Fc-fragments as well as most of the other antigens tested including hen egg lysozyme and keyhole limpet hemocyanin. M-O never bound to *M. tub.* HSP60; RAB-A and M-P sometimes did, indicating an instability of the epitope detected in this assay (not shown). None of the three MAbs bound to gelatine or DNA (not shown).

Binding inhibition assays

In an ELISA also low affinity reactivities are detected. In contrast, in the immunofluorescence assay the antibody will bind preferentially to those epitopes which react with the

Table 1. Affinities of MAbs

Antigen	K_D (M)		
	M-O	RAB-A	M-P
Desmin	$\mathbf{0.1 \times 10^{-7}}$	1.9×10^{-7}	52.0×10^{-7}
Actin	n.d.	$\mathbf{0.1 \times 10^{-7}}$	15.0×10^{-7}
Keratin K8	n.d.	5.2×10^{-7}	12.5×10^{-7}
Collagen Typ II	n.d.	4.1×10^{-7}	3.5×10^{-7}
KLH	n.d.	2.4×10^{-7}	$\mathbf{1.4 \times 10^{-7}}$

Dissociation constants (K_D) were determined by inhibition with the same antigen in solution as used as coat in the ELISA. n. d. = not determined, as M-O only bound to desmin. In the same way the K_D of the commercial MAb K8.13 binding to keratin K8 was determined (2.3×10^{-7} M). For each MAb the lowest K_D (highest affinity) is indicated by boldface type.

Table 2. Binding inhibition measured by cytometric analyses

Inhibitor	MFI (% Inhibition)	
	M-O	RAB-A
non	400	320
desmin	70 (100%)	130 (84.4%)
desmin (1:10)	90 (98.4%)	230 (37.5%)
actin	n.d.	80 (100%)
keratin K8	150 (79.4%)	280 (16.7%)
collagen Type II	n.d.	220 (41.7%)
cartilage extract	180 (69.8%)	210 (45.8%)
M. tub. HSP 60	n.d.	90 (96.9%)
Controls:		
isotype control + anti-Ig^{FITC}	85	80
anti-Ig^{FITC}	70	70

The antibodies were used in a concentration of 2.5 μg/ml (M-O) and 7 μg/ml (RAB-A), respectively. Inhibitors were used in the highest concentration used in the ELISA inhibition assays ($3.7 - 6 \times 10^{-7}$ M). Desmin in addition was used in a 10× lower concentration. Inhibition was calculated as $100 \times [100 - (MFI_{+Inhibitor} - MFI_{isotype\ control}/(MFI_{-inhibitor} - MFI_{isotype\ control})]$. n. d. = not determined.

highest affinity. This might explain certain discrepancies between both assays, e. g. binding of RAB-A to actin in the ELISA but not producing a "typical" actin-binding staining pattern on HEp-2 cells. We therefore measured the binding affinities for the most interesting antigens according to a protocol by FRIGUET et al (28). Within the range of concentrations tested, inhibition could not be obtained with a number of antigens, including IgG-Fc-fragments, albumins, lysozyme and transferrin (not shown). The binding of the MAbs to these antigens therefore did only occur with low affinity. Homologous inhibition could be obtained with desmin (all 3 MAbs) and with actin, keratin K8, collagen

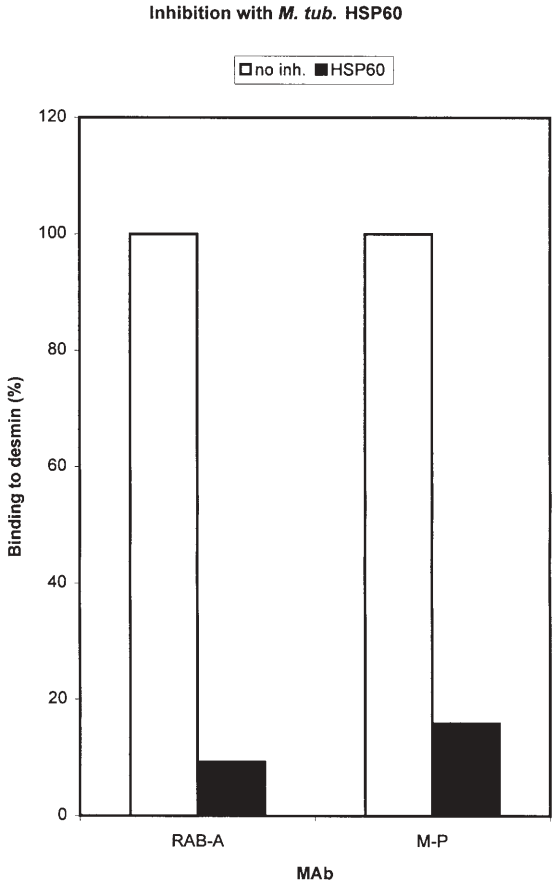


Figure 4. Specific binding of M-P and RAB-A to *M. tub.* HSP60 in solution. The binding of RAB-A and M-P to desmin was inhibited by soluble *M. tub.* HSP60. Bound MAbs were detected with goat-anti-human-IgM^{AP} or -IgA^{AP} and PNPP as substrate.

type II and KLH (RAB-A and M-P). The affinities calculated from these data are shown in Table 1. Vimentin was not available for inhibition assays, and therefore could not be included here. To confirm a high affinity binding of RAB-A and M-P to *M. tub.* HSP60, we performed a heterologous inhibition assay using desmin as coating antigen and HSP60 as soluble inhibitor, which showed reproducibly a strong inhibition (Fig. 4).

In addition to ELISA inhibition assays, we studied the inhibition of the binding to cytoskeletal antigens of HEp-2 cells by cytometry. MAbs were preincubated in appropriate concentrations with the soluble antigens, and then tested for binding to permeabilized cells (Table 2). Binding of M-O and RAB-A to HEp-2 cells was inhibited with desmin in a concentration-dependent way. Interestingly, M-O was also inhibited by keratin K8 and by a cartilage extract, containing only proteoglycans. As might be expected from the affinity measurements, actin was the best inhibitor for RAB-A, followed by *M. tub.* HSP60 and desmin, whereas keratin K8 was of lower efficiency. Interestingly, RAB-A binding to HEp-

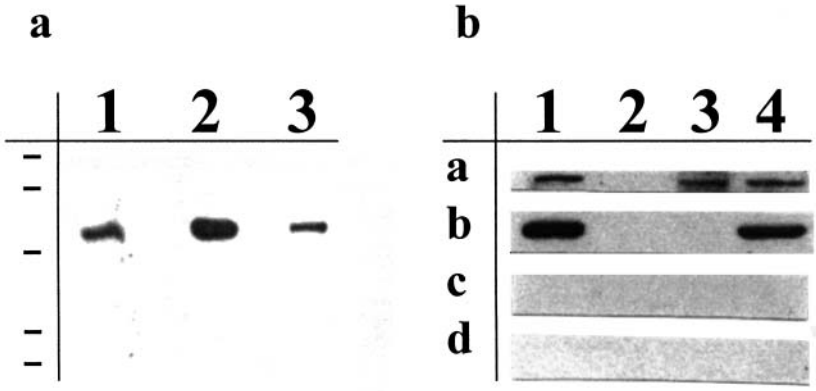
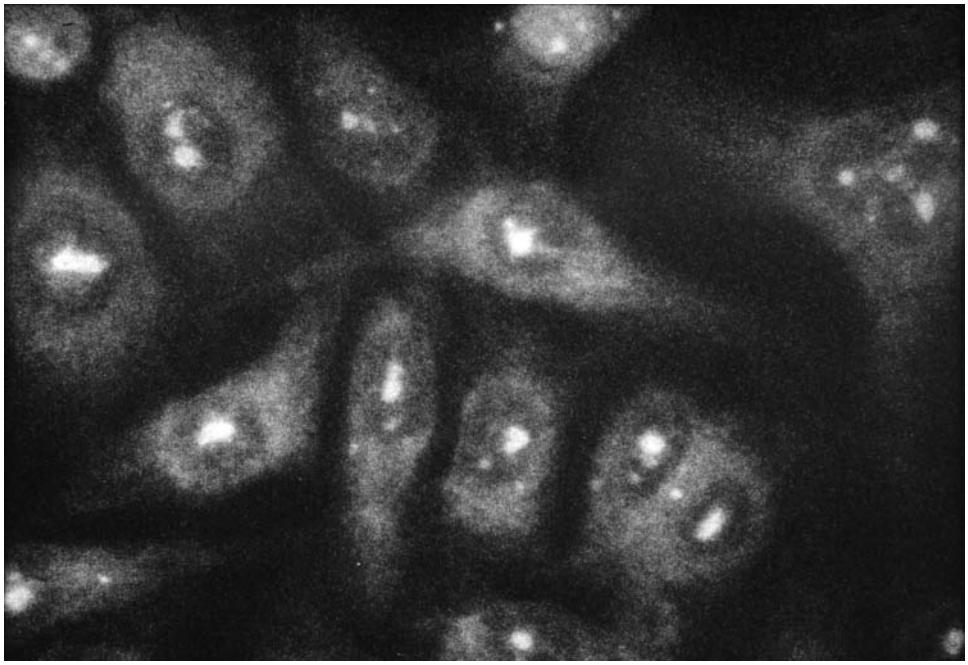


Figure 5. Binding of MAbs to desmin and to proteins extracted from HEp-2 cells detected by immunoblotting. Desmin (a) or proteins solubilized from HEp-2 cells (b) were applied to SDS-PAGE followed by immunoblotting. M-P (lane a1), M-O (lane a2) and RAB-A (lane a3) bound to desmin (detection with anti-IgM or anti-IgA, respectively). Binding to proteins extracted from HEp-2 cells by the consecutive application of lysis buffers and sonication as described in Materials and Methods is demonstrated for M-O (b). The lanes contained extract 1 (lane b3), extract 2 (lane b2), extract 3 (lane b1) or a mixture of all 3 extracts (lane b4). Blots were first incubated with M-O (a) or the isotope control (c), then stripped and retested with antibodies specific for vimentin (b) or desmin (d). The bands detected in (a) and (b) were identical.

2 cells was also inhibited by collagen type II and the collagen-free cartilage extract. The binding of the commercial antibody K8.13 was completely inhibited by keratin K8 only.

Binding to proteins extracted from HEp-2 cells

All three MAbs were selected for analysis because they stained HEp-2 cells in a specific pattern. The cytoskeleton of HEp-2 cells is supposed to contain type I and II intermediate filaments (IMFs), the type III IMF vimentin, but not desmin, and microfilaments containing actin. The cytoskeletal staining patterns we observed showed similarities to patterns obtained with antibodies exclusively binding to vimentin or keratins. The specificities of RAB-A and M-P detected in ELISA and in the inhibition assays suggest that the binding to either vimentin or keratins or to both molecules produced the specific cytoskeletal staining pattern of these two antibodies with different fine specificities. The cytoskeletal binding of M-O was reflected in the ELISAs by binding to desmin, in the binding inhibition assays in addition by binding to keratin K8. M-O did not bind to vimentin in the ELISA. All three MAbs bound to desmin in immunoblots (Fig. 5a), but not to keratin K8 or actin (not shown), indicating that they are able to recognize certain but not all epitopes on denatured molecules. We therefore prepared protein extracts from HEp-2 cells, using 3 different buffers with increasing capacity to solubilize cytoskeletal and nuclear proteins, and analysed these in immunoblots. Here we show that M-O bound to a major band with an apparent MW of 65 kD, present in 2 of the 3 protein extracts, and in their mixture. However, only in 1 of the extracts the same band was also detected by a MAb specific for vimentin (Fig. 5b). A MAb specific for desmin did not bind to any protein in these HEp-2 extracts (Fig. 5b). Thus, most probably the cytoskel-



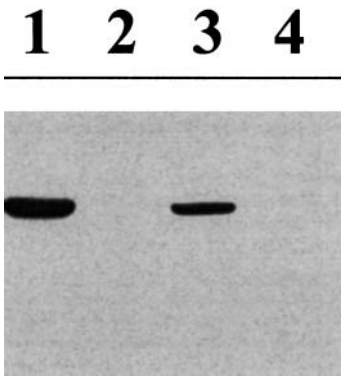


Figure 6. Binding of serum IgG (patient M) to HEp-2 cells and vimentin. Serum from patient M (diluted 1:50) stained HEp-2 cells in indirect immunofluorescence detected with anti-IgG^{FITC} (a). HEp-2 protein extract 3 was immunoprecipitated with serum and *Staph. aureus* protein A, coupled to sepharose beads. The immunoprecipitate and controls were tested by SDS-PAGE and immunoblots, using anti-vimentin or an isotype control antibody. The extract contained vimentin (lane 1, detection with anti-vimentin), as did the immunoprecipitate with IgGs from patient M (lane 3). The controls were negative (lane 2, control precipitate, anti-vimentin; lane 4, immunoprecipitate, isotype control antibody).

etal staining pattern observed with M-O was due to binding to vimentin. In addition, at least one other protein – of the same size – might be recognized.

Serum antibodies of similar specificity detected in the patient

Serum IgG as well as IgM (not shown) from patient M stained HEp-2 cells in indirect immunofluorescence with a similar pattern as M-O and RAB-A, especially regarding the nucleolar staining (Fig 6a). In addition, proteins extracted from HEp-2 cells were immunoprecipitated with the serum and *Staph. aureus* protein A. The analysis in immunoblots demonstrated the presence of vimentin in these immunoprecipitates (Fig. 6b).

Discussion

Three MAbs derived from two patients with RA and rPC, respectively, were studied for their specific binding capacities in several assay systems to further understand how the appearance of such antibodies might be related to disease. These MAbs were chosen because they showed similar staining patterns on HEp-2 cells, but differed in other respects. One of them (RAB-A) is a human monoclonal IgA and therefore can be expected to be derived from a B cell which *in vivo* was selected and driven by antigen. One of the IgMs (M-O) produced a very similar immunofluorescence staining pattern on HEp-2 cells as did RAB-A. The other IgM (M-P) was derived from the same EBV-transformed B cell line as M-O, and showed a related but different specificity. The results of this study are summarized in Table 3.

The data illustrate that one antibody may show different crossreactivities dependent on the assay system used. The differences concern mainly the availability of the epitopes recognized, i. e. most probably their stability under different conditions. As an example, binding of M-O to desmin could be detected in all assays; desmin thus contains an epitope not sensitive to conformational changes (solid phase, soluble or denatured). The reactivity with vimentin, however, was not detected in ELISA, although vimentin most probably was the molecule detected by the staining of HEp-2 cells which do not contain desmin.

Crossreactions between desmin and vimentin have been described before and might be expected as both molecules belong to the same protein family and are highly homologous (more than 60% identity (18,29)). Also murine and human MAbs reacting with desmin and nuclear antigens were described, and it was suggested that the nuclear antigens might be DNA (30–33). We do not yet know the identity of the nucleolar epitope detected by M-O and RAB-A. However, both MAbs did not bind to DNA, neither in ELISA, nor in the Crithidia assay (not shown). The data clearly show that one antibody may stain HEp-2 cells with two distinct patterns, i. e. react with cytoskeletal and nucleolar epitopes, although the exact molecules detected in the nucleolus are still not identified.

In addition to recognizing desmin and vimentin, all three antibodies bound to keratin K8; RAB-A and M-P also to actin. Nevertheless, the immunofluorescence pattern observed on HEp-2 cells did not resemble a typical actin-specific staining, although especially RAB-A bound to actin with high affinity ($K_D = 0.1 \times 10^{-7}$ M). None of the antibodies reacted with actin or keratin K8 when these molecules were denatured as in SDS-PAGE. In immunoblots with protein extracts M-O bound to a band also detected by an antibody specific to vimentin. In addition – and to our surprise – M-O also bound to a yet undefined protein of the same apparent molecular weight present in an extract not expected to contain cytoskeletal components.

Most interesting are the crossreactions of RAB-A and M-P with *M. tub.* HSP60 and all three MAbs with cartilage antigens, which were detected in ELISA and/or in inhibition assays. RAB-A and M-P bound to human collagen type II, with relative high affinities ($K_D = 4.1 \times 10^{-7}$ M and 3.5×10^{-7} M, respectively). M-O did not bind to collagen, however inhibition assays were not performed. All three MAbs reacted with an antigen present in the collagen-free cartilage extract. Some preliminary evidence suggests this to be cartilage link protein (34). HSP60 molecules have been implied in arthritis models, and the titers of antibodies binding to HSP60 molecules might be enhanced in patients with RA (35–37). Furthermore, crossreactions between HSP60 and cartilage components appear to play a role in the induction of experimental arthritis (35, 38).

Table 3. Summary of the results

Assay	Human MAbs		
	M-O	RAB-A	M-P
Immunofluorescence on HEp-2 cells: binding pattern	Cytoskeletal and nucleolar epitopes	Cytoskeletal and nucleolar epitopes	Cytoskeletal epitopes
ELISA: binding assay[1]	Specific: desmin	Polyreactive: desmin and others	Polyreactive: desmin and others
ELISA: inhibition assay	Desmin Others not tested	Actin, desmin, KLH, collagen type II, keratin K8, (*M. tub.* HSP60)[2]	KLH, collagen type II, keratin K8, actin, desmin, (*M. tub.* HSP60)[2]
Cytometry: inhibition	Desmin, keratin K8, proteoglycans	Actin, desmin, proteoglycans, collagen type II, keratin K8 (*M. tub.* HSP60)	Desmin Others not tested
SDS-PAGE/Immunoblot: binding	Desmin HEp-2 protein extract: vimentin, additional protein	Desmin HEp-2 protein extract: vimentin, additional protein	Desmin HEp-2 protein extract:vimentin

[1] Only positive reactions are listed
[2] Listed in the order of affinities. (Affinitiy measurement was not possible for HSP60)

Affinities of human autoreactive antibodies were only rarely reported in the literature (39–44). Especially, affinity measurements of autoantibodies reacting with cytoskeletal autoantigens were still missing. The affinities we measured are relatively high and place the MAbs RAB-A, M-O and M-P well within the group of antibodies induced by experimental immunizations. This is exemplified by the K_D measured for the commercially available murine antibody specific for keratin K8 (2.3×10^{-7} M). MAbs derived from newborn mice, which were regarded as "natural autoantibodies" because they bound to a variety of self antigens including actin and DNA, reacted with K_Ds in the range of 3×10^{-5} to 5×10^{-6} M (11). These data are directly comparable as all reports used the same method for affinity measurements (9, 11, 28, 39–44). However, as affinities of "natural autoantibodies" fall into a broad range (K_Ds between 5×10^{-3} M and 5×10^{-11} M), affinity cannot serve to define an antibody as "natural" – in addition an unmutated v-gene is required.

Mono- or polyreactivity in ELISA does not relate to affinity (9). RAB-A and M-P showed a very similar binding profile in ELISA, whereas M-O specifically only bound to desmin. The highest affinity for desmin was measured for M-O ($K_D = 0{,}1 \times 10^{-7}$ M) closely followed by RAB-A ($K_D = 1.9 \times 10^{-7}$ M), whereas M-P bound with 500fold lower affinity to desmin than M-O ($K_D = 52 \times 10^{-7}$ M).

To our knowledge this is the first compilation of human MAbs describing their fine specificities to different antigens in several assay systems as well as their binding affinities. We provide evidence that the staining patterns detected in the HEp-2 immunofluorescence assay routinely employed in many rheumatology laboratories can be produced by antibodies binding with high affinity to autoantigens relevant for the disease (cartilage) as well as foreign antigens (mycobacterial HSP60). To the best of our knowledge human MAbs showing these crossreactivities have not been described before.

The data do not answer the question whether these antibodies or the B cells synthesizing them are involved in the pathogenesis of the disease. As shown here, the blood of patient M contained not only the B cells from which M-O and M-P derived, but also an IgG and IgM population with similar binding characteristics. It is therefore well conceivable, that these were induced in a similar way as the antibodies binding to cytoskeletal antigens in rats developing arthritis after immunization with mycobacterial HSP60 (12).

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References

1. BRADWELL, A. R., R. P. STOKES, and G. D. JOHNSON. 1995. Atlas of HEp-2 patterns, KNP Group Ltd. Redditch, 118 pp.
2. BORG A. A., P. T. DAWES, and D. L. MATTEY. 1993. Increased levels of IgA antibodies to cytokeratin-18 and epidermal keratin in rheumatoid arthritis. Arthr. Rheum. **36:** 229.
3. JACOBS R., M. G. BUTLER, and D. L. SCOTT. 1990. Antibodies to cardiolipin and intermediate filaments: A study of autoimmunity in rheumatoid arthritis. Clin. Rheumatol. **9:** 509.
4. KATAAHA P. K., S. M. MORTAZAVI-MILANI, G. RUSSELL, and E. J. HOLBOROW. 1985. Anti-intermediate filament antibodies, antikeratin antibody, and antiperinuclear factor in rheumatoid arthritis and infectious mononucleosis. Ann. Rheum. Dis. **44:** 446.

5. KURKI O., T. HELVE, and A. I. VIRTANEN. 1983. Antibodies to cytoplasmic intermediate filaments in rheumatic diseases. J. Rheumatol. **10:** 558.
6. MAYET W., E. HERMANN, M. BACHMANN, M. MANNS, and K.-H. MEYER ZUM BUESCHENFELDE. 1990. Correlation of anti-cytoskeleton antibody activities in synovial fluid with interleukin-6 in patients with osteoarthritis and inflammatory joint disease. Klin. Wochenschr. **68:** 685.
7. OLIVER J. M., J. L. SENÉCAL, and N. L. ROTHFIELD. 1985. Autoantibodies to the cytoskeleton in human sera, In: Shay JW. ed. Cell and Muscle Motility, New York: Plenum Press 55.
8. OSUNG O. A., M. CH. and R. A., and E. J. HOLBOROW. 1982. Antibody to intermediate filaments of the cytoskeleton in rheumatoid arthritis. Ann. Rheum. Dis. **41:** 69.
9. AVRAMEAS S. 1991. Natural autoantibodies – from 'horror autotoxicus' to 'gnothi seauton'. Immunol. Today **12:** 154.
10. CASTELL, M. 1996. Cytoskeletal autoantibodies. In: Peter JB & Shoenfeld Y. ed. Autoantibodies. Amsterdam:, Elsevier Science 217.
11. TERNYNCK T., and S. AVRAMEAS. 1986. Murine natural monoclonal autoantibodies: A study of their polyspecificities and their affinities. Immunol. Rev. **94:** 99.
12. FRANCH A., C. CASTELLOTE, J. L. VILA, S. VILARO, and M. CASTELL. 1994. Anticytoskeletal autoantibody development in Adjuvant Arthritis. J. Rheumatol. **21:** 489.
13. NAPARSTEK Y., and P. H. PLOTZ. 1993. The role of autoantibodies in autoimmune disease. Ann. Rev. Immunol. **11:** 79.
14. HASKARD D. O., V. GUL, A. MORGAN, P. KATAAHA, N. A. SZAINES, and J. R. ARCHER. 1985. Human monoclonal antibodies from patients with rheumatoid arthritis:crossreactions against cellular constituents. Clin. Exp. Immunol. **61:** 496.
15. LANDENBERG P.V., R. RZEPKA, and I. MELCHERS. 1999. Monoclonal autoantibodies from patients with autoimmune diseases: Synovial fluid B lymphocytes of a patient with rheumatoid arthritis produced an IgG antibody recognizing J-sequences of Igκ chains in a conformation dependent way. Immunobiol. **200:** 205.
16. LANDENBERG P.V., C.V. LANDENBERG, M. GRUNDDL, G. SCHMITZ, J. SCHÖLMERICH, and I. MELCHERS. 1999. A new antigenic epitope localized within human kappa light chains specific for RA and SLE. J. Autoimmunity **13:** 83.
17. STEINERT P. M., and D. R. ROOP. 1988. Molecular and cellular biology of intermediate filaments. Annu. Rev. Biochem. **57:** 593.
18. FUCHS E., and K. WEBER. 1994. Intermediate filaments: Structure, dynamics, function, and disease. Ann. Rev. Biochem. **63:** 345.
19. MCADAM L. P., M. A. O'HANLAN, R. BLUESTONE, and C. M. PEARSON. 1976. Relapsing polychondritis. Prospective study of 23 patient and a review of the literature. Medicine **55:** 193.
20. ARNETT F. C., S. M. EDWORTHY, and D. A. BLOCH, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthr. Rheum. **31:** 315.
21. KURZIK-DUMKE U., C. SCHICK, R. RZEPKA, and I. MELCHERS. 1999. Overexpression of human homologues of the bacterial DnaJ chaperone in the synovial tissue of arthritis patients. Arthr. Rheum. **42:** 210.
22. KEMPIS J. V., and G. KIENZLE. 1998. VCAM-1 (CD106) on primary human articular chondrocytes: functional regulation of expression by cytokines and comparison with ICAM-1 (CD54) and VLA-2. Arthr. Rheum. **41:** 1296.
23. GRUNOW R., S. JAHN, and T. PORSTMANN, et al. 1988. The high efficiency, human B cell immortalizing heteromyeloma CB-F7. J. Immunol. Meth. **106:** 257.
24. YAN T., H. BURKHARDT, T. RITTER, and F. EMMRICH. 1992. Specificity and T cell receptor β chain usage of a human collagen type II-reactive T cell clone derived from a healthy individual. Eur. J. Immunol. **22:** 51.
25. MELCHERS I., J. JOOSS-RÜDIGER, and H. H. PETER. 1997. Reactivity patterns of synovial T-cell lines derived from a patient with Rheumatoid Arthritis. I. Reactions with defined antigens and autoantigens suggest the existence of multireactive T cell clones. Scand. J. Immunol. **46:** 187.
26. KOGA T., A. WAND-WÜRTTEMBERGER, J. DERBRUYN, M. E. MUNK, B. SCHOEL, and S. H. E. KAUFMANN. 1989. T cells against a bacterial heat shock protein recognize stressed macrophages. Science **245:** 1112.

27. RUDOLPHI U., A. HOHLBAUM, B. LANG, H. H. PETER, and I. MELCHERS. 1998. The B cell repertoire of patients with rheumatoid arthritis. Frequencies and specificities of peripheral blood B cells reacting with human IgG, human collagens, a mycobacterial heat shock protein and other antigens. Clin. Exp. Immunol. **92:** 404.
28. FRIGUET B., A. F. CHAFFOTTE, L. DJAVANDI-OHANIANCE, and M. E. GOLDBERG. 1985. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Meth. 305.
29. VARON D., S. LINDER, E. GEMBOM, L. GUEDG, H. LANGEHEIM, A. BERREBI, and Z. ESHHAR. 1990. Human monoclonal antibody derived from an Autoimmune Thrombocytopenic Purpura patient, recognizing an intermediate filament's determinant common to vimentin and desmin. Clin. Immunol. Immunopathol. **54:** 454.
30. SENÉCAL J. L., and J. RAUCH. 1988. Hybridoma lupus autoantibodies can bind major cytoskeletal filaments in the absence of DNA-binding activity. Arthr. Rheum. **31:** 864.
31. KAMEI H. 1990. Location of nuclear antigen(s) recognized by DSB389 MAb, a monoclonal antibody against desmin, observed by confocal laser scanning fluorescence microscopy. Cell Biol. Int. Rep. **14:** 727.
32. ICHIKI S., STEMARIE, and J. L. SENÉCAL. 1992. Cross-reactive and non cross-reactive subsets of autoantibodies to intermediate filament proteins: Analysis of sera from 379 connective tissue patients and controls. Arthr. Rheum. **35:** R42 (Abstract).
33. KAMEI H. 1996. Nuclear Antigens, as DNA, of DSB389 MAB and some other Anti-Desmin Monoclonal Antibodies. Cell Biol. Intern. **20:** 769.
34. MENGE, T., HUMMES, R., SCHLEDZ, M., LANGENBERG, U., PETER, H. H., and I. MELCHERS. 1995. Functional characterization of human monoclonal antibodies with specificities related to rheumatoid arthritis. Immunobiol. **194:** 145. (Abstract).
35. FEIGE U., and W. VAN EDEN. 1996. Infection, autoimmunity and autoimmune disease. EXS **77:** 359.
36. HANDLEY H. H., M. D. NGUYEN, D. T. YU, R. S. GUPTA, and J. H. VAUGHAN. 1995. Purification of recombinant human Hsp60: use of a GroEL-free preparation to assess autoimmunity in rheumatoid arthritis. J. Autoimmunity **8:** 659.
37. RUDOLPHI U., R. RZEPKA, S. BATSFORD, S. H. E. KUAFMANN, K. VON DER MARK, H. H. PETER, and I. MELCHERS. 1997. The B cell repertoire of patients with rheumatoid arthritis. II. Increased frequencies of IgG^+ and IgA^+ B cells specific for mycobacterial heat-shock protein 60 or human type II collagen in synovial fluid and tissue. Arthr. Rheum. **40:** 1409.
38. VAN EDEN W., J. HOLOSHITZ, Z. NEVO, A. FRENKEL, A. KLAJMAN, and I. R. COHEN. 1985. Arthritis induced by a T-lymphocyte clone that responds to mycobacterium tuberculosis and to cartilage proteoglycans. Proc. Natl. Acad. Sci. USA **82:** 5117.
39. CASALI P., and A. L. NOTKINS. 1989. CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunol. Today **10:** 364.
40. EHRENSTEIN M. R., C. LONGHURST, and D. A. ISENBERG. 1993. Production and analysis of IgG monoclonal anti-DNA antibodies from systemic lupus erythematodes (SLE) patients. Clin. Exp. Immunol. **92:** 39.
41. EHRENSTEIN M. R., C. LONGHURST, and D. A. ISENBERG. 1992. Production and analysis of human monoclonal antibodies to myeloperoxidase. Immunol. **76:** 617.
42. LU E. W., M. DEFTOS, H. TIGHE, D. A. CARSON, and P. P. CHEN. 1992. Generation and characterization of two monoclonal self-associating IgG rheumatoid factors from a rheumatoid synovium. Arthr. Rheum. **35:** 101.
43. LU E. W., and M. DEFTOS. 1993. Generation and molecular analysis of two rheumatoid arthritis synovial fluid derived IgG rheumatoid factors. Arthr. Rheum. **36:** 927.
44. DIAW L., C. MAGNAC, O. PRITSCH, M. BUCKLE, P. M. ALZARI, and G. DIGHIERO. 1997. Structural and affinity studies of IgM polyreactive natural autoantibodies. J. Immunol. **158:** 968.

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