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Macrophage-lysis mediated by autoantibodies to heat shock protein 65/60

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Abstract

Macrophages in atherosclerotic lesions have been shown to express high amounts of heat shock protein 60 (hsp60), a highly conserved protein. Patients with atherosclerosis have high titers of anti-hsp65/60 antibodies (Ab) recognizing macrophages in the lesions. To elucidate the role of anti-hsp65/60 Ab in macrophage cytotoxicity, human high titer serum and purified anti-hsp65/60 Ab were tested on in vitro heat-stressed cells of a human macrophage cell line (U937) and macrophages derived from peripheral blood. Application of heat stress at 42°C for 30 min resulted in marked upregulation of hsp60 mRNA, followed by increased protein expression as determined by Northern blot and FACS-analyis, respectively. Compared to unstressed cells, both high titer serum and anti-hsp65/60 Ab preferentially bound to the surface of stressed U937 macrophages, but not control antibodies. Furthermore, high titer serum and anti-hsp65/60 Ab exerted significant ($P < 0.01$) complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) on stressed ^{51}Cr-labelled U937 and peripheral blood derived macrophages. Thus, macrophages expressing hsp60 can be lysed by autoantibodies against hsp65/60, which may contribute to cell death in atherosclerotic plaques in vivo. Copyright © 1997 Elsevier Science Ireland Ltd.

Keywords: Antibodies; Atherosclerosis; Autoimmunity; Cell death; Heat shock protein; Stress proteins

1. Introduction

Heat shock protein (hsp) 60 is essentially involved in the maintenance of undisturbed protein folding and -translocation, which confers protection against various environmental stresses [1,2]. High temperatures and various other stimuli, including cytokines, heavy metal anions, oxidative and mechanical stress, lead to rapid upregulation of hsp60 synthesis. Because of the high amino acid sequence homology from prokaryotes to man, e.g. human hsp60 shows over 50% homology to its mycobacterial homologue hsp65, it has been postulated that these proteins are involved in a variety of autoimmune conditions and diseases, as well as being inducers of atherosclerosis in rabbits [3,4]. Increased hsp expression has been found in human atherosclerotic lesions by several investigators [5–7], the most intensive of which is noted in macrophages around the necrotic core of the plaque.

Our previous epidemiological study showed a correlation between the occurrence of atherosclerotic lesions in carotid arteries and elevated serum antibodies against hsp65 independent from classical atherosclerosis risk factors, such as hypercholesterolemia, hyperten-

Abbreviations: ADCC, Antibody dependent cellular cytotoxicity; hsp, heat shock protein.

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sion, smoking and diabetes [8]. Western blot analyses using recombinant human hsp60 and homogenized human plaque tissue containing abundant hsp60 demonstrated high cross-reactivity of anti-hsp65 antibodies with the human stress protein homologue [9]. Positive binding was observed, when plaque sections were stained with these antibodies.

The advanced atherosclerotic lesion contains a necrotic core surrounded by lipid-laden macrophages, and rupture of these lesions causes the acute clinical manifestations. The detailed mechanism by which the necrotic core forms, remains to be elucidated. Recent studies [10,11] focused on apoptosis in atheroma provided evidence that 10 to 46% of macrophages were TUNEL (terminal deoxynucleotidyl transferase-(TdT) mediated dUTP-biotin nick end labeling) positive, indicating an apoptotic state. Because many factors, including high temperature, tumor necrosis factor-α, and oxidative stress leading to cell necrosis and/or apoptosis, induce hsp expression in the cells [12,13], it is of interest whether an immunological reaction to hsp expressed on macrophages contributes to cell death in vitro. To address this issue, we performed experiments with a human macrophage tumor cell line, U937, and with differentiated macrophages from peripheral blood monocytes using high titer antiserum and a purified anti-hsp Ab. We provide evidence that anti-hsp65/60 Abs positively stain the surface of heat-stressed macrophages and mediate cell cytotoxicity in the presence of complement and effector cells.

2. Material and methods

2.1. Human serum samples and antibody determination

Based on a previous clinical study examining cardiovascular risk factors in 867 clinically healthy subjects from Bruneck, Italy (8, Table 1), 20 samples of human sera were selected according to their anti-hsp65 antibody titer and the occurrence of atherosclerotic plaques in carotid arteries. Sonographic evaluation of carotid arteries for atherosclerotic lesions has been extensively described elsewhere [14].

Table 1
Anti-hsp65/60 Ab titers

Probe	Antibody titer (ELISA)
High titer antisera No. 1–10	>1:1280
High titer serum pool	1:1280
Low titer antisera No. 1–10	<1:40
Low titer serum pool	1:20
Purified anti-hsp65/60 antibodies	1:640
Unbound Ig fraction	<1:10
mAbs (anti-α-actin, -FSH, -CD3)	<1:10
Guinea pig serum (source of complement)	<1:10

Hsp65/60-Abs were determined with an ELISA.

An enzyme-linked immunosorbent assay (ELISA), based on a previously established method [8], was used to determine hsp65 antibody titers. Briefly, ELISA-plates (Petra-Plastic, Würzburg, Germany) coated with 0.1 μg recombinant hsp65/well (Stressgene Biotechnologies, Victoria, BC) were incubated with serially diluted human sera. To detect bound antibodies, horseradish peroxidase (HRP)-conjugated rabbit anti-human Ig (cat. No. 217, Dako, Copenhagen, Denmark) was used and visualized with its substrate 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma, Munich, Germany). Absorbance at 410 nm was quantified by a Microelisa Reader and was considered positive when extinction exceeded 0.2.

2.2. Affinity chromatography of anti-hsp65/60 antibodies

Specific antibodies against hsp65 were purified from human high titer sera by using a chromatography column packed with 3 mg recombinant mycobacterial hsp65. Details, including protein coupling, serum preparation and affinity chromatography have been reported previously [14].

Briefly, total immunoglobulin from heat-inactivated, pooled human high titer sera was precipitated by 50%-saturated ammonium sulfate. Imunoglobulins were then transferred to a chromatography column containing 2 ml hsp65-precoupled agarose gel (Affi-Gel 15-Kit, cat. No. 153-6051, Biorad, Hercules, CA). After incubating for 30 min and rinsing to remove unbound immunoglobulin, anti-hsp65/60 Abs were eluted by adding 20 mM HCl. A total amount of 3.3 mg anti-hsp65/60 antibodies was purified from 12 ml high titer human serum. Antibody specificity was verified by either Western blotting or semiquantitative ELISA measurement. The unbound fraction of precipitated Ig was used as a negative control Ab, free of hsp65/60 reactivity.

2.3. Cell culture

For cultivation of U937 macrophages, 1×10^6 cells were seeded into a 25 ml flask (Falcon, Becton Dickinson, Oxnard, CA) and cultivated in 10% FCS/RPMI 1640 supplemented with 1% L-glutamine and 1% penicillin-streptomycin in 5% CO_2 at 37°C. Cell concentrations did not exceed 3×10^6 macrophages/ml medium, and fresh medium was supplied every 3 days.

Human monocytes were isolated from peripheral blood of healthy donors by density centrifugation (Lymphoprep, Nycomed Pharmaceuticals, Oslo, Norway) and subsequent adherence to a plastic surface for

30 min at 37°C. Cells were used as primary cultures and grown in 10% FCS/RPMI 1640 supplemented with 1% L-glutamine and 1% penicillin-streptomycin in 5% CO_2 at 37°C. According to a standard protocol [15], differentiation of U937 cells and peripheral blood monocytes was induced by addition of 1000 U/μl granulocyte-colony stimulating factor (G-CSF, Roche) and granulocyte/macrophage colony stimulating factor (GM-CSF, Sandoz), respectively, for at least 48 h.

2.4. Immunoglobulin-subclass determination

The distribution of Ig-classes within anti-hsp65/60 Ab was measured by quantitative radial immunodiffusion, as described previously [14]. IgG subclasses were assessed by a sandwich-ELISA using a commercially-available test kit (cat. No. MK001, The Binding Site, Birmingham, UK). Specifically-bound antibodies were detected by adding an anti-human IgG peroxidase conjugate and its substrate, 3,3,5,5-tetramethylbenzidine (TMB). Assessment of IgA subclasses was performed by the same technique using monoclonal Abs to IgA1 (cat. No. 1170279, Boehringer Mannheim, Germany) and IgA2 (cat. No. 1170252, Boehringer Mannheim) respectively, and, for quantification, an immunoglobulin standard (cat. No. BP062, The Binding Site) with known IgA-subclass concentrations.

2.5. RNA isolation and Northern blot

Total RNA was isolated following a standard protocol described earlier [16]. RNA (10 μg/lane) was denatured with formaldehyde (Merck, Darmstadt, Germany), electrophoresed in a 1% agarose gel and transferred onto a nylon membrane (zeta Probe, Biorad, Richmond, CA). RNA was UV-crosslinked in a UV Stratalinker (Stratagene, La Jolla, CA) and hybridized with the ^{32}P-labelled probe (*Bam*H1–*Eco*R1 fragment of human heat shock protein 60 gene; clone pSJ60 was a gift from S. Jindal, Cambridge, MA) and glyceraldehyde-3 phosphate dehydrogenase (GAPDH, [17]) as a control at 65°C. The filters were then washed and exposed to Kodak XAR films at -80°C.

2.6. Western blot

To induce hsp60 expression, cultivated U937 macrophages (5×10^6) received 30 min of heat stress at 42°C followed by 3 h of recovery at 37°C. Control cells (5×10^6) remained at 37°C. Cells were then washed twice in PBS, pelleted by centrifugation at $1200 \times g$ for 10 min and resuspended in 200 μl of lysis buffer (0.15 M NaCl, 50 mM Tris, 1 mM EDTA, 0.05% sodium dodecylsulfate, 0.5% Triton X-100 and 1 mM phenylmethylsulfonylfluoride, pH 7.4) for 1 h. The cell lysate was then centrifuged at $5000 \times g$ for 10 min, the supernatants harvested and analyzed for their protein content by a protein assay kit (cat. No. 500-0006, Bio-Rad, Hercules, CA). For electrophoresis, total cell proteins were dissolved (1:2 vol/vol) in sample buffer containing 5% β-mercaptoethanol, 15% glycerol, 3% SDS, 0.1 M Tris, pH 6.8. Protein samples, including recombinant human hsp60 (derived from clone PKK 13A, [18]), recombinant mycobacterial hsp65 and total macrophage proteins from both stressed and unstressed cells, were separated on a 12% polyacrylamide gel under reducing conditions. Proteins were electrophoretically blotted onto nitrocellulose membranes (BA85, Schleicher and Schuell, Dassel, Germany) in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3, at 100 mA for 12 h. Membranes were blocked with 1% BSA (cat. No. A3912, Sigma)/PBS for 1h and 2% non fat dry milk for another hour, then probed with human high-or low titer antiserum (dilution 1:500 v/v in 1% BSA/PBS), purified anti-hsp65/60 Ab (100 ng/ml) or unbound Ig-fraction (100 ng/ml) for 2 h. A HRP-conjugated rabbit anti-human Ig (cat. No. Z259, Dako, dilution 1:400 v/v) was utilized as detection antibody and the reaction visualized by 4-chloro-1-naphtol/hydrogen peroxide (Sigma).

2.7. FACS-analysis

Human U937 macrophages were heat stressed at 42°C for 30 min followed by 3 h of recovery at 37°C, or kept at 37°C without further treatment. For flow cytometric analysis, cells (5×10^5/tube) were washed twice in PBS, resuspended in 100 μl PBS supplemented with 1% BSA (Sigma) and appropriately diluted antibodies (human high- and low titer antiserum, anti-hsp65/60 antibody, unbound Ig-fraction, mAb anti-hsp60 (ML-30) and mAb anti-human CD3 control) and incubated for 1 h at room temperature. As detection antibodies, a FITC-conjugated rabbit anti-human Ig (cat. No. F200, Dako) or—rabbit anti-mouse Ig (cat. No. F261, Dako), respectively, were added for another 30 min. Cells were analyzed immediately after washing 3 times in PBS. To exclude unspecific binding of first and second antibody, each incubation step was preceded by blocking the macrophage Fc-receptors with normal rabbit serum (dilution 1:5 v/v) for 20 min. Absorption experiments were assessed by preincubation of anti-hsp65/60 Ab with either 50 μg soluble recombinant human hsp60 or 50 μg ovalbumin, as a control. Fluorescence measurements were performed by FACS analysis (FACScan, Becton Dickinson). Details of FACS settings and methods of quantifying fluorescence intensity of labelled cells have been described previously [19].

To measure cytoplasmic expression of hsp60, cells were fixed with 2% paraformaldehyde (Merck) for 10 min, washed 3 times in PBS and then permeabilized with 0.025% nonyl-phenoxy-polyethoxy-ethanol (NP-

40, Sigma) in PBS for 10 min. The permeabilized cells were incubated with the monoclonal anti-hsp60 Ab II-13, reacting with an epitope between aa288–366 [18] or, for control purpose, with a mAb to human FSH (a gift from P. Berger, Innsbruck) as first antibodies.

2.8. Cytotoxicity assays

To induce hsp60 expression, native or differentiated U937-macrophages were heat stressed as described above, whereas control cells remained at 37°C. After a recovery time of 90 min and washing twice, 1×10^6 each stressed and unstressed cells were radioactively labelled by addition of 200 μl 10% FCS/RPMI containing 100 μCi ^{51}chromium (Behringwerke) at 37°C for another 90 min. Free radioactivity was removed by three further washes in RPMI and 2×10^4 cells resuspended in 50 μl medium (10% FCS/RPMI) were subsequently transferred to U-bottom 96-well microtiter plates (Falcon). Thereafter, 50 μl of diluted antibody (dilution of anti-hsp65/60 Ab 1:20, high titer serum adjusted to same anti-hsp65/60 titer and control Ab to same protein concentration as anti-hsp65/60 Ab) and, for complement-mediated cytotoxicity, another 50 μl of guinea pig serum (cat. No. 1140, Behringwerke) as a source of complement were added to the culture. Guinea pig serum was selected to be free of anti-hsp65/60 Ab, as determined by ELISA. Adherent differentiated macrophages derived from peripheral blood were treated equally, heat stress and radioactive labeling (5 μCi ^{51}Cr/well) were directly performed in the 96-well plate. The probes were incubated for 14 h at 37°C until the assay was stopped by addition of 100 μl ice-cold RPMI. Cells were then pelleted at $300 \times g$ for 10 min and 150 μl supernatant was harvested for analysis of radioactivity in a gamma counter (Wallac-Wizzard Automatic Gamma Counter, Helsinki, Finland).

To determine antibody-dependent cellular cytotoxicity (ADCC), peripheral blood mononuclear cells (PBMC) from healthy blood donors were used as effectors instead of complement. PBMC were isolated by density centrifugation as described above. Following an assay protocol similar to that descibed for complement-mediated cytotoxicity, 100 μl 10% FCS/RPMI with PBMC in a 10/1 to 100/1 effector/target ratio were then added to the cultures and incubated for 7 h.

Both human, including low titer antiserum and unbound Ig-fraction, and monoclonal mouse Abs, including mAb anti-α-actin (cat. No. 1148818, Boehringer, Mannheim, FRG) and mAb anti-CD3 (cat. No. M756, Dako) were used as controls in the cytotoxicity experiments. Blocking experiments were performed by addition of anti-hsp65/60 Ab preincubated with 50 μg soluble recombinant hsp60 or alternatively 50 μg ovalbumin for 1 h at 37°C.

Table 2
Ig-subclasses of purified anti-hsp65/60 antibodies

Subclasses	Normal serum[a]	hsp65 antiserum	anti-hsp65/60 Abs
IgGl	52.5–72.6	60.7	33.4
IgG2	20.1–30.1	26.8	38.6
IgG3	5.2–7.0	8.8	7.7
IgG4	0.2–11.8	3.7	20.1
IgAl	75–93	95	41
IgA2	7–25	5	59

IgG- and IgA-subclasses (% of total) were determined by ELISA and calculated against standard.
[a] Values for Ig-subclasses in nomal human serum from the Diagnostic Laboratory of The Immunopathology Unit of the Institute for General and Experimental Pathology, University of Innsbruck, Medical School.

Specifically released radioactivity was determined by calculation according to the formula: (release in the presence of antibodies − spontanous release)/(maximal release − spontaneous release). Maximal release was obtained by adding 1% Triton X-100 to the cultures. Spontaneous release was determined in the presence of effectors (complement or PBMC) but without antibodies and did not exceed 15% of maximal release. Statistical analysis was performed by using a paired Student's t-test.

3. Results

Twenty human sera, half from subjects with carotid atherosclerosis and high titer anti-hsp65/60 serum antibodies (high titer sera), and half from healthy age and sex matched control subjects (low titer sera), were used either pooled or, in the case of high titer serum, used as an affinity purified anti-hsp65/60 Ab preparation. Analysis of Ig subclass distribution of anti-hsp65/60 Abs revealed a strong preponderance of IgG_4 and IgA_2 subclasses compared to a standard serum pool. Since anti-hsp65/60 Abs constitute only a minor part of total immunoglobulins, this shift in Ig subclasses does not affect the composition of total Ig in the original high titer serum (Table 2).

As a prerequisite for enhanced surface binding and cytotoxicity of anti-hsp65/60 Ab to stressed macrophages, we studied the hsp60-mRNA and protein expression and the binding of anti-hsp65/60 Ab to the newly synthesized hsp60 of macrophages.

3.1. Induction of hsp60 at the mRNA level

Following an initial heat shock at 42°C for 30 min, hsp60-mRNA expression was assessed after different recovery times. A weak upregulation of transcription

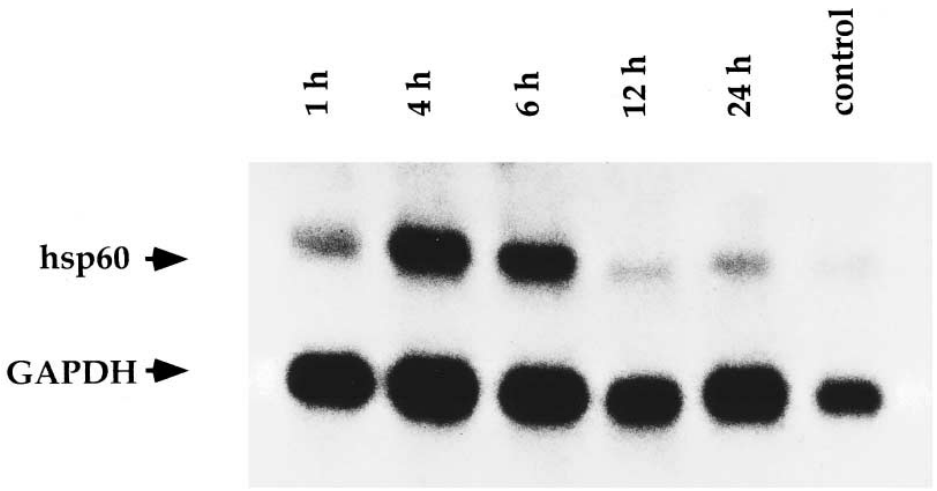

Fig. 1. Hsp60-mRNA expression (Northern blotting). U937-macrophages were heat stressed at 42°C for 30 min, recovered at 37°C for various time intervals (1–24 h) and then analyzed for hsp60- and GAPDH-mRNA by Northern blotting (10 μg RNA/lane). While GAPDH-mRNA levels were fairly constant throughout the experiment, hsp60-mRNA expression increased to maximal levels within 4 h, followed by a decrease to almost baseline levels within 12 h after heat stress.

(or of mRNA stability) was observed as early as 1 h after heat shock, but the major mRNA level response was observed between 1–6 h following heat shock. Signals decreased to almost baseline levels within 24 h (Fig. 1).

3.2. Induction of hsp60 at the protein level

The extent of intracellular hsp60 expression of heat stressed and unstressed U937 macrophages was assessed by flow cytometric measurement of fixed, permeabilized cells (Fig. 2). Hsp60 expression was almost undetectable in unstressed cells (1, mean fluorescence intensity (MFI) $2.5 + 4$), while heat stress induced an up to 10-fold increase of specific fluorescence intensity, as detected by anti-hsp60 mAb II-13 (2, MFI 82 ± 21). Cells stained with an isotype-matched, control Ab (anti-FSH) revealed no difference in fluorescence intensity in response to 42°C heat stress (3, MFI 8 ± 5; 4, MFI: 10 ± 3). This low basal, but strongly heat-inducible, hsp60 expression of U937 cells was also confirmed by immunoblotting (data not shown).

3.3. Binding of anti-hsp65/60 Ab to hsp60 of macrophages

To demonstrate the recognition of human macrophage hsp60 by anti-hsp65/60 antibodies, total lysate proteins of both stressed and unstressed U937 cells were probed with high titer serum or anti-hsp65/60 Ab, respectively. For control purposes, binding of these Abs to recombinant mycobacterial hsp65 and human hsp60 was also assessed (Fig. 3).

Corresponding to earlier data, specific antibodies purified from anti-hsp65 high titer sera recognized mycobacterial hsp65 (a) and also showed a strong cross-reaction to its human homologue hsp60 (b). Furthermore, when total lysate protein from heat-stressed (42°C for 30 min) macrophages was probed with high titer serum (c) or anti-hsp65/60 Ab (d), binding to a 60 kDa band was observed, whereas this band was absent in unstressed cells. Low titer antiserum (e) and unbound Ig-fraction (f), which contained very little or no anti-hsp65/60 Abs (Table 1), did not evoke a similar staining with protein lysates of stressed or unstressed cells, although a weak binding to two unidentified low molecular weight proteins was observed in pooled low titer serum.

3.4. Surface reactivity of anti-hsp65/60 Ab

To test for possible surface binding of anti-hsp65/60 Abs to human macrophages, stressed and unstressed (Fig. 4) living U937 cells were stained with both hsp65/60-reactive and non-reactive Abs and assessed for surface fluorescence intensity by FACS-analysis. The viability of labelled macrophages was determined by FACS scatter analysis (always $> 95\%$), and only living cells were gated and subjected to immunofluorescence measurements. According to the protocol described above, macrophages were heat stressed at 42°C for 30 min to allow induction of hsp60 expression, while control cells were not stressed. Surface binding on heat-stressed macrophages labelled with high titer serum (hts, MFI 557 ± 86) or anti-hsp65/60 Abs (hspAb, MFI 341 ± 65) showed an up to three-fold increase (two-fold in the case of high titer serum) compared to unstressed cells. Unstressed cells were not stained by anti-hsp65/60 Ab (MFI 129 ± 21), but were stained by high titer serum to a considerable extent (MFI 301 ± 34). This

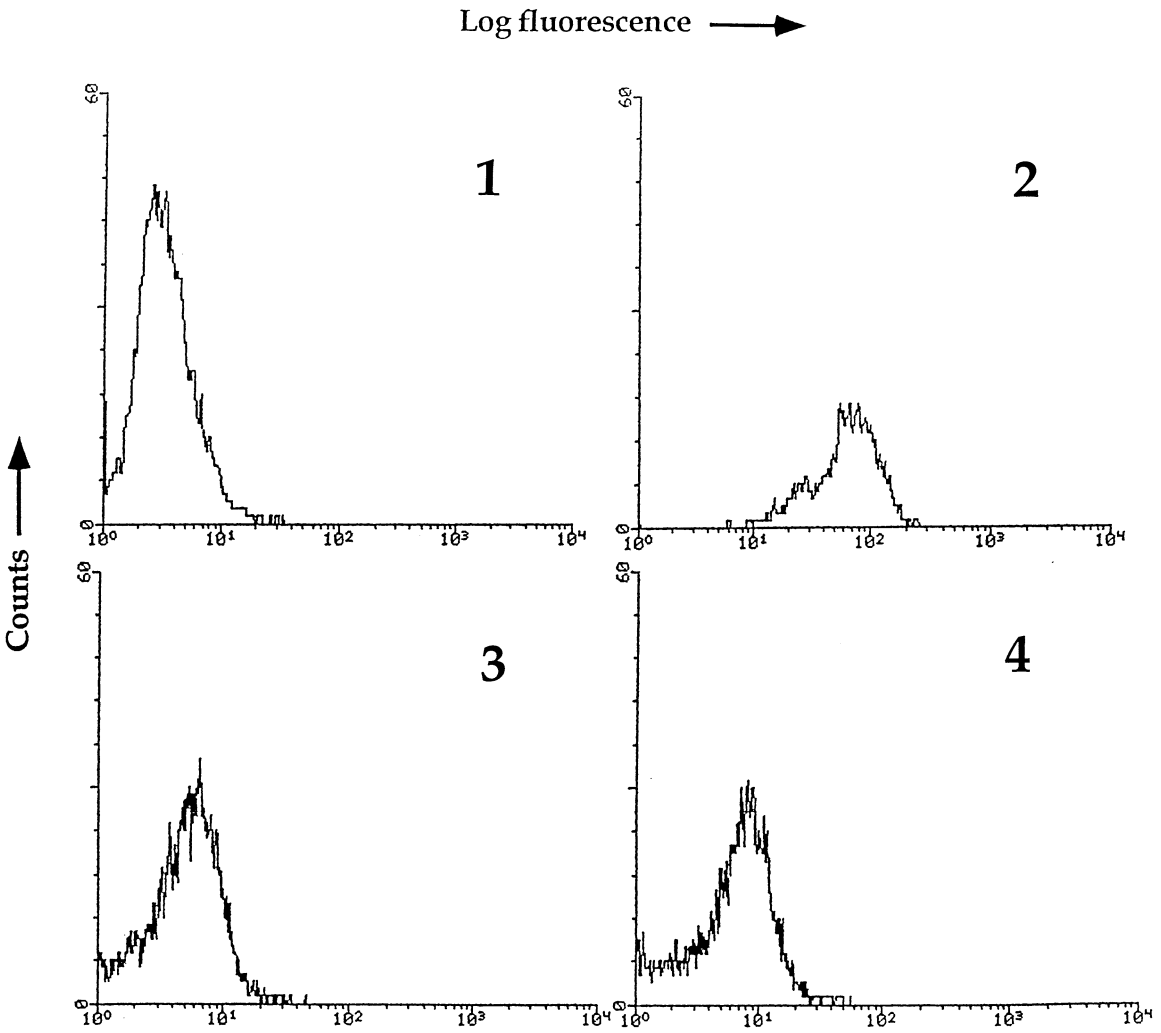

Fig. 2. FACS-analysis of intracellular hsp60 expression. U937-macrophages were heat-stressed at 42°C for 30 min following recovery at 37°C for another 4 h (2, 4) or left without heat treatment (1, 3). Thereafter, both stressed and unstressed cells were fixed and permeabilized to allow for intracellular staining of hsp60. As first antibodies, anti-hsp60 mAb II-13 (dil. 1:20 v/v; (1, 2) or anti-FSH mAb (dil. 1:20 v/v (3, 4)) were used and reaction was then visualized by FITC-conjugated anti-mouse Ig (dil. 1:40 v/v). Note the increased fluorescence intensity of stressed macrophages stained by the mAb II-13 (2). Quantitative values: (1), MFI $2.5 + 4$; (2), MFI 82 ± 21; (3), MFI 8 ± 5; (4), 10 ± 3.

may be explained by the presence of other autoreactive Abs in the high titer serum pool recognizing antigens different from hsp60 but binding to the macrophage surface independent from any effect related to heat stress. Binding was almost abolished by preincubation of anti-hsp65/60 Ab with 50 μg recombinant hsp60 (hsp block, MFI 35 ± 16) but not with 50 μg ovalbumin (OA block, MFI 328 ± 31). Control Abs, including low titer serum (lts, MFI 104 ± 9 and 74 ± 19, respectively), unbound Ig-fraction (uIg, MFI 102 ± 20 and 50 ± 31, respectively) and mouse-mAb anti-CD3 (CD3, MFI 68 ± 41 and 72 ± 35, respectively), all of them lacking anti-hsp65/60 reactivity, did not bind to stressed or unstressed cells. Confirming earlier observations [20], the mouse-mAb ML-30, specific for hsp60, did not stain the macrophage surface (ML-30, MFI 87 ± 11 and 69 ± 13, respectively), indicating a different hsp60-epitope on the macrophage surface recognized by human serum Ab.

3.5. Complement-mediated cytotoxicity

When ^{51}Cr-labelled U937-macrophages were heat stressed, recovered and incubated in the presence of high titer serum or anti-hsp65/60 Ab and complement, cells were lysed to a significant degree by antibody and complement (Fig. 5a). In comparison, the cytotoxic effect of high titer serum and anti-hsp65/60 Ab upon unstressed macrophages was significantly ($P < 0.01$) lower, but still present. Control antibodies, such as low titer serum, unbound Ig-fraction and mAbs to α-actin and to CD3, evoked no complement-mediated cytotoxi-

city on either stressed and unstressed macrophages. Analysis of the time-kinetics of complement-mediated cytotoxicity revealed that cell damage entailed by anti-hsp65/60 Abs was measurable after 6 h and peaked after 18 h (data not shown). In addition, the cytotoxic response was demonstrated to be strongly dependent on the antibody dose applied (Fig. 5b).

The cytotoxic effect of high titer serum and anti-hsp65/60 Ab to G-CSF/GM-CSF differentiated U937 cells (Fig. 7) was even stronger; especially on heat stressed cells a high degree of cytotoxicity was observed ($P < 0.01$). However, also the lysis of unstressed cells exceeded that of undifferentiated U937 cells. Assessing differentiated macrophages from peripheral blood, the cytotoxic effect of anti-hsp65/60 Ab (Fig. 8a) was much more prominent, entailing almost 80% of specific release. Furthermore the effect was specifically blockable by preabsorption with 50 μg recombinant hsp60.

3.6. Antibody dependent cellular cytotoxicity

Stressed and unstressed ^{51}Cr-labelled U937-macrophages were cultivated in the presence of Abs and PBMC as effectors to assess ADCC. Again, hsp65/60-reactive Abs, including serum and purified Ab, evoked a significant ^{51}Cr release via ADCC, whereas unstressed cells were lysed to a far lesser extent (Fig. 6a; $P < 0.01$). Furthermore, the cytotoxic effect of high titer serum and anti-hsp65/60 Ab was demonstrated to be strongly dependent on the amount of effector cells (data not shown) and the concentration of antibodies applied (Fig. 6b). The observation in some experiments that a certain degree of lysis also occurred with unstressed cells and anti-hsp65/60 Abs may be explained by the fact that in vitro cultures constitute a stressful condition by themselves. Low titer serum, unbound Ig-fraction, mAb anti-α-actin and mAb anti-CD3 showed no measurable ADCC on stressed or unstressed cells.

Again, anti-hsp65/60 Ab exhibited a far higher degree of cytotoxicity on differentiated macrophages from peripheral blood, with up to 90% of specific cell lysis (Fig. 8b). Blocking by preincubation of the Ab with 50 μg recombinant hsp60 led to a 50% reduction of cell lysis (Fig. 8b).

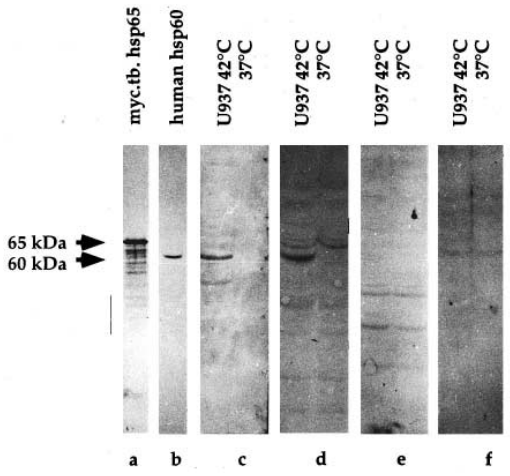

Fig. 3. Hsp-Ab binding to human macrophages (Western blotting). Recombinant mycobacterial hsp65 (a, 0.5 μg/lane), recombinant human hsp60 (b, 1 μg/lane) and total lysate protein from 42°C heat-stressed (c–f, 50 μg/lane) and unstressed (37°C, c–f, 50 μg/lane) U937-macrophages were separated on a 12% SDS-PAGE under reducing conditions and blotted on a nitrocellulose membrane. Blots were probed with anti-hsp65/60 Ab (a,b,d; 100 ng/ml), high titer serum (c; 1:500 v/v), low titer serum (e; 1:500 v/v) or not bound Ig-fraction (f; 100 ng/ml). The reaction was visualized by a rabbit anti-human Ig peroxidase conjugate and its substrate 4-chloro-1-naphthol/H_2O_2. Anti-hsp65/60 Abs recognize hsp65 (a), hsp60 (b) and a 60 kDa band of stressed U937 macrophages (d). The weak staining at approximately 65 kDa on both stressed and unstressed lysate protein is not specific.

4. Discussion

We have found that autoantibodies to heat shock protein 65/60 are associated with carotid [8] and coronary atherosclerosis [21]. In this study we demonstrate binding, surface staining and cytotoxic activity of these disease-associated antibodies on macrophages, cells known to play key roles in the atherogenic process.

Infiltration of macrophages has shown to be one of the earliest events occurring during atherogenesis [22]. In addition to their well-established role in scavenger receptor-mediated endocytosis of lipoproteins and modifications thereof, macrophages are also pivotal for immune reactions in the diseased vessel wall, as illustrated both by their colocalization with T lymphocytes and by their early production of cytokines, such as TNFα and IL-1, by these cells [23]. Both lipoprotein uptake and inflammatory processes have been shown to essentially contribute to plaque formation and are potential inducers of hsp via their cytokine products. Furthermore, immunocytochemical investigations of early and late atherosclerotic lesions revealed a high expression of hsp60 [5] and hsp70 [6] by macrophages themselves, with the strongest expression in macrophages around the necrotic core of advanced lesions, where the cells are likely to face many stressors. In this case, the hsp expression may serve to protect against possible deleterious attacks [24], e.g. oxidative damage by the incorporated material.

Several different factors, including mechanical and chemical stressors, may be involved in the induction of both hsp60 and hsp70 in lesion macrophages. Firstly, oxidative stress by altered lipoproteins seems to play an important role, since oxidized LDL is abundantly found in human atherosclerotic lesions and is known as a potent activator of monocytic stress protein synthesis

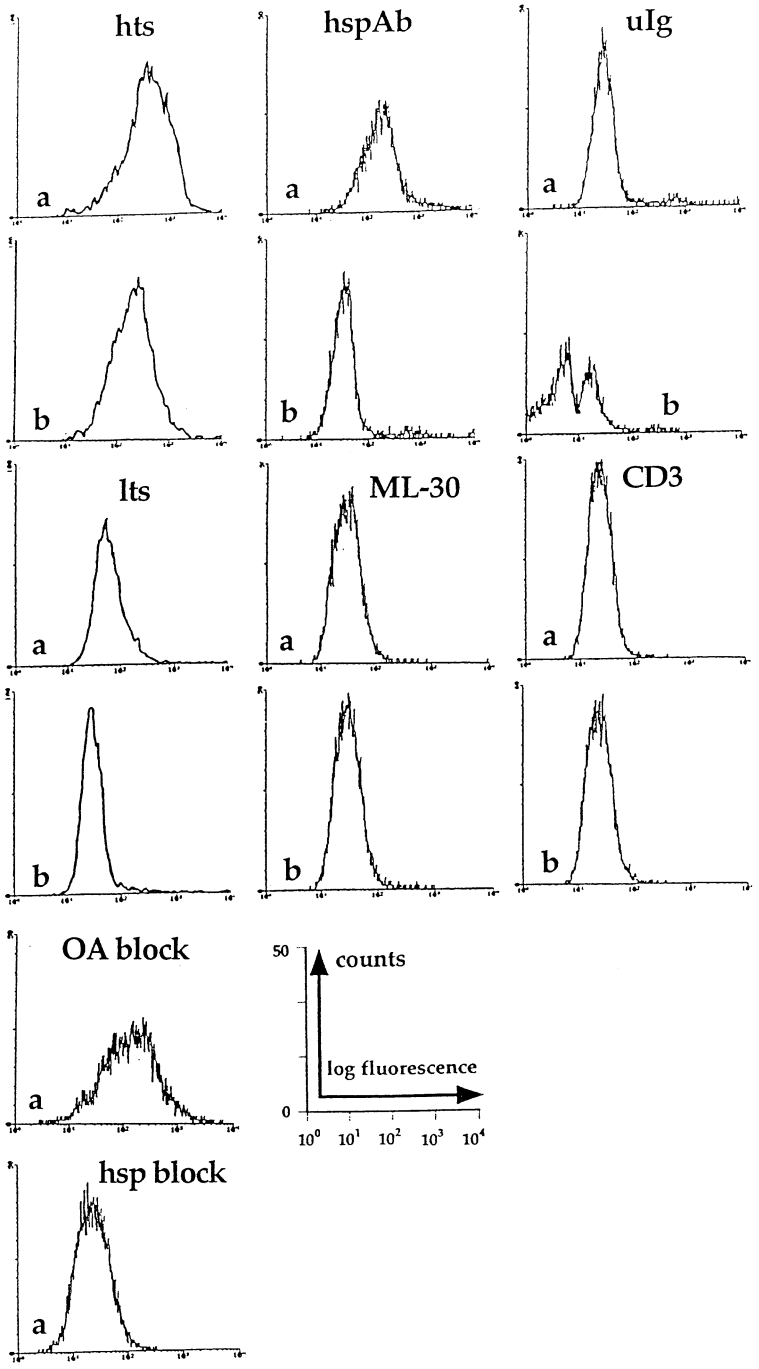

Fig. 4. Surface binding of hsp-Ab to human macrophages (FACS-analysis). 1×10^6 U937 macrophages were heat stressed at 42°C for 30 min (a) or left without treatment at 37°C (b). After recovering for 3 h at 37°C, washing and blocking the Fc-receptors by normal rabbit serum (dilution 1:5 v/v, 20 min) living cells were incubated with the following antibodies for 1 h: high titer serum (hts; dil. 1:200 v/v), low titer serum (lts; dil. 1:200 v/v), anti-hsp65/60 Ab (hspAb; dil. 1:50 v/v), unbound Ig-fraction (uIg; dil. 1:50 v/v), mAb ML-30 (ML-30; dil. 1:20 v/v), mAb anti-human CD3 (CD3; dil. 1:50 v/v). After a further blocking step with normal rabbit serum (20 min) the reaction was visualized by the use of a FITC-conjugated anti-human Ig (dil. 1:40 v/v, 30 min). Cells were immediately subjected to FACS analysis. Surface staining only occurred on stressed and, weaker, unstressed macrophages stained with high titer serum, and on stressed cells stained by the anti-hsp65/60 Ab. Binding was almost abolished by preincubation of anti-hsp65/60 Ab with 50 μg recombinant hsp60 (hsp block, MFI 35 ± 16) but not with 50 μg ovalbumin (OA block, MFI 328 ± 31). Mean + S.D. values are from three diferent experiments: hts, high titer serum 557 ± 86 and 301 ± 34); hspAb, anti-hsp65/60 Ab (MFI 341 ± 65 and 129 ± 21); OA block, anti-hsp65/60 1:50 preabsorbed with 50 μg ovalbumin (328 ± 31); hsp block, anti-hsp65/60 1:50 preabsorbed with 50 μg recombinant human hsp60 (35 ± 16); lts, low titer serum (104 ± 9 and 74 ± 19); uIg, unbound Ig-fraction (102 ± 20 and 50 ± 31); ML-30, mouse-mAb ML-30, (87 ± 11 and 69 ± 13); and CD3, mouse-mAb anti-CD3 (68 ± 41 and 72 ± 35).

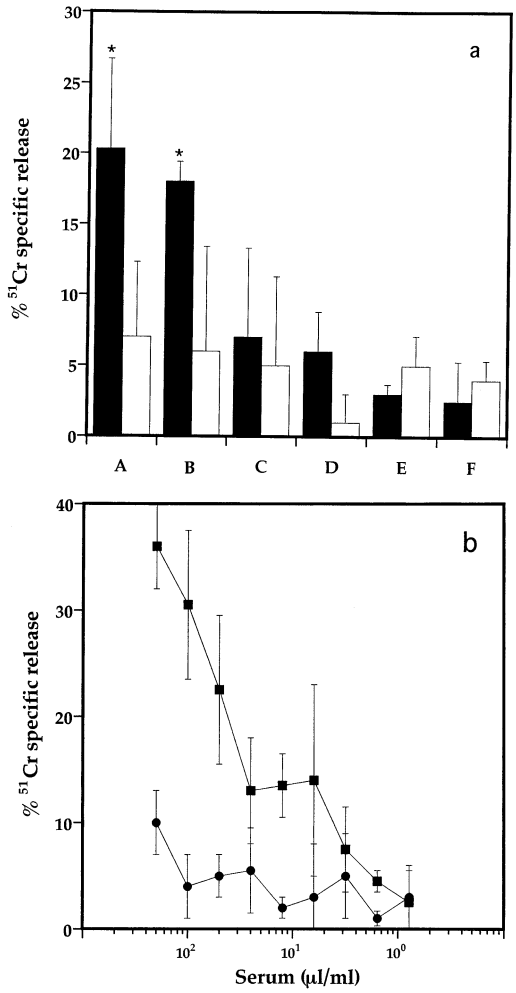

Fig. 5. Complement mediated cytotoxicity. 2×10^6 U937 macrophages were heat-stressed at 42°C for 30 min followed by 90 min of recovery or left without stress at 37°C. The cells were then washed and labelled with 100 μCi ^{51}Cr in 200 μl 10% FCS/RPMI for another 90 min. 2×10^4 Labelled cells were subsequently seeded into each well of a 96-well microtiter plate and incubated in the presence of antibodies (a: dilution 1:20, b: serial dilution) and guinea pig complement in a total assay volume of 150 μl for 14 h at 37°C. Fig. 5a: antibodies, pooled high titer serum (A), anti-hsp65/60 Ab (B), pooled low titer serum (C), unbound Ig-fraction (D), mAb anti-α-actin (E), and mAb anti-CD3 (F) were probed on both stressed (■) and unstressed (□) cells. Values are means of 3 independent experiments and show specific cytotoxicity induced in the presence of Abs. Fig. 5b: Dose response curve. Serially diluted high (■) or low (●) titer serum were probed on stressed U937 macrophages in the presence of complement. Values are means of three independent experiments and indicate specific release in the presence of antibodies. * Indicates a P-value < 0.01.

[25,26], both by the way of free radical production [12,27] and by its phagocytosis, which also induces hsp. Secondly, hypoxia which is considered a powerful inducer of hsp, might be increasingly important in the core region, particularly when the lesion thickens and nutrition becomes critical [28,29]. Thirdly, cytokine production is a common and early feature of all cells participating in atherogenesis, and TNFα and IL-1 also trigger hsp induction in addition to a variety of proinflammatory changes [13,30]. In this context, it is noteworthy that hsp65 itself can induce TNFα and IL-1 production in human monocytes [31], which could point to the establishment of a vicious cycle in stress protein expression of intimal macrophages. Fourthly,

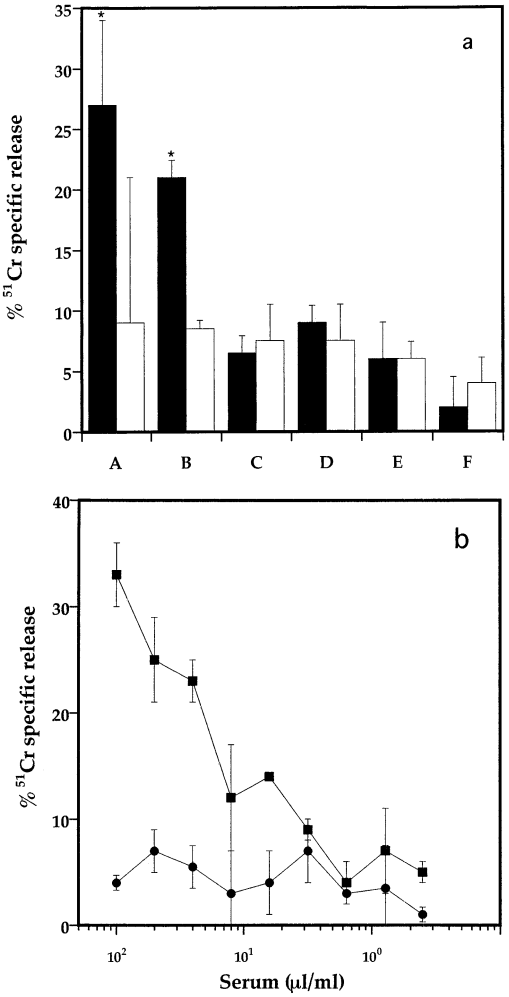

Fig. 6. ADCC. U937 macrophages were heat stressed, as described earlier or kept unstressed. After 90 min of recovery the cells were labelled with 100 μCi ^{51}Cr in 200 μl 10% FCS/RPMI for 90 min and 2×10^4 cells were subsequently aliquoted in each well of a 96-well microtiter plate. The assay was started by addition of antibodies (a: dilution 1:20, b: serial dilution) and effector cells (1×10^6, effector/target ratio 50:1) in a total volume of 200 μl. After 7 h of incubation free radioactivity was determined in a γ counter and specific release calculated. Fig. 6a: A: high titer serum; B: anti-hsp65/60 Ab; C: low titer serum; D: unbound-Ig fraction; E: mAb anti-α-actin; F: mAb anti-CD3 probed on stressed (■) and unstressed cells (□). * Indicates a P-value < 0.01. Fig. 6b: serially diluted high (■) or low (●) titer serum were probed on stressed U937 macrophages in the presence of PBMC (effector/target ratio 50:1). Values are means of three independent experiments and indicate specific release in the presence of antibodies. * P-value < 0.01.

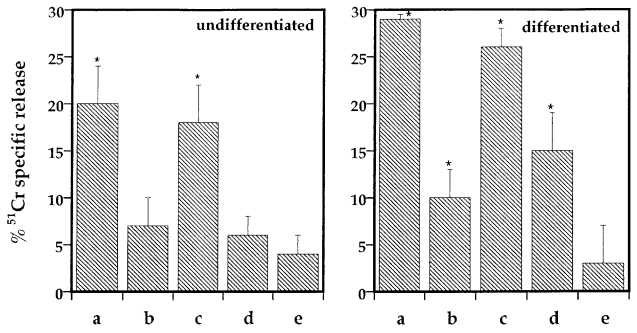

Fig. 7. Complement-mediated cytotoxicity: comparison of differentiated and undifferentiated U937 cells. U937 monocytes were induced to differentiate by the presence of 1000 U/μl G-CSF and GM-CSF, respectively, for at least 48 h (right), or were kept undifferentiated (left). Cells were subsequently heat stressed (a,c) at 42°C for 30 min or cultivated without heat stress (b,d) and radioactively labelled according to the protocol described above. 2×10^4 Labelled cells were transferred into each well of a 96-well microtiter plate and incubated in the presence of antibodies (dilution 1:20) and guinea pig complement in a total assay volume of 150 μl for 14 h. Supernatants were analyzed for free radioactivity in a γ counter. a,b: High titer serum; c,d: anti-hsp65/60 Ab; e: control antibody (anti-CD3 mAb).

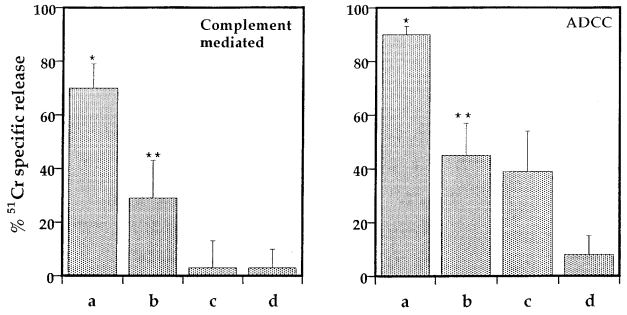

Fig. 8. Differentiated peripheral blood derived macrophages: complement-mediated cytotoxicity and ADCC. Peripheral blood derived monocytes, cultivated on a 96-well plate, were driven to differentiation by the addition of 1000 U/μl G-CSF and GM-CSF, respectively, for at least 48 h at 37°C. Heat stressed (a,b,d) and unstressed (c) macrophages were radioactively labelled by the addition of 5 μCi/well ^{51}Cr, washed and incubated with anti-hsp65/60 Ab (a + c), anti-hsp65/60 Ab preabsorbed with 50 μg recombinant human hsp60 (b) or anti-CD3 mAb as control (d). Addition of effectors, assay time and evaluation of results are according to the protocol described for U937 cells. Values are means of three independent experiments. *, ** Indicate a P-values < 0.01. Note the significant (**) blocking effect by addition of recombinant hsp60 (b) to the test.

differentiation of blood monocytes to tissue macrophages during their transmigration to the intima is likely accompanied by hsp induction [32]. It should be noted here, that differentiation of monocytes to macrophages in vitro rendered the cells much more susceptible to a cytotoxic attack of anti-hsp65/60 Ab. An explanation for this phenomenon and also for elevated cytotoxicity to unstressed differentiated cells, may be the hsp60 induction during differentiation and subsequent recognition of the antibody.

Originally, proteins of the hsp60 familiy were considered to be located only intracellularly in mitochondria, where they facilitate protein translocation and protect the protein from harmful enzymatic attacks during folding. However, evidence in the past few years points to an additional surface location of hsp60, or portions thereof, on the plasma membrane of various cells, including tumor cells [33,34], mononuclear [35,36] and endothelial cells [37]. Although the exact mechanism of hsp60 transportation to the cell surface, and its possible function there, remain to be clarified, hsp might preserve the integrity of plasma membrane proteins. Other mechanisms that could explain surface expression of hsp include trafficking of hsp peptides by assembly with MHC class I and II proteins, binding of circulating hsp from the outside to the plasma membrane, and detection of an immunological crossreaction of hsp65/60 antibodies with a highly homologous other plasma membrane protein.

In this study, both complement mediated cytotoxicity and ADCC against stressed hsp60-expressing macrophages were observed upon addition of anti-hsp65/60 antibodies. The presence of immunoglobulin deposits and immunocomplexes (both complement activators) is unique to the atherosclerotic lesion and is not found in healthy vascular walls [38]. Furthermore, complement activation followed by generation of the lytic C5–9 complex and expression of monocytic complement receptors for C3b and C3bi [39] has been confirmed by a variety of studies investigating atherosclerotic lesions. Interestingly, we found a strong shift in the immunoglobulin class- and sub-class distribution within the hsp65/60 antibody population compared to normal serum, as reflected by high levels of IgM- (see Ref. [14]), IgA (IgA_2)- and IgG_4 antibodies. IgM is known to form immunocomplexes and represents a potent activator of the complement cascade, whereas IgG4 can mediate ADCC through its binding to the Fc-receptor. IgG4 autoantibodies have also been described in systemic sclerosis [40] and rheumatoid arthritis [41], both of which may be hsp-associated autoimmune diseases. The elevation of IgA- antibodies cannot be explained by local immunity, since local production in the intima is ruled out due to the absence of B-cells [5,42]. However, increased concentrations of IgA-serum antibodies have been shown to be associated with a variety of chronic inflammatory processes, including atherosclerosis [43].

Regardless of the main effector mechanism involved, cytotoxicity of anti-hsp65/60 antibodies could lead to areas of severe cell death and necrosis, especially at macrophage-rich and highly stressed areas of the atherosclerotic lesion. This might explain phenomena, such as formation of the necrotic core [44] and plaque rupture, which are known to coincide with abundant monocytic infiltration [45]. For example, the known in

vitro cytotoxic effect of oxidized LDL [46] may be related to their ability to induce hsp and to the presence of hsp-antibodies in vivo. Alternatively, hsp65/60 antibodies may weaken the arterial cell wall in general, facilitating an attack of other plaque-associated elements which entails cell death and the formation of a necrotic core.

The principal mechanisms of the humoral immune response against hsp have been reported previously [9,47]. Hsp65/60 antibodies could either represent a cross-reactive product of the host response against bacterial hsp or could be due to a bona fide primary autoimmune response to altered self-hsp expression [48]. The epitopes on hsp65/60 involved in this humoral immune reaction are now under investigation in our laboratory [49].

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