OxLDL immune complexes activate complement and induce cytokine production by MonoMac 6 cells and human macrophages

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Abstract **Oxidized low density lipoprotein (OxLDL) is immunogenic and induces autoimmune responses in humans. OxLDL antibodies are predominantly of the proinflammatory IgG1 and IgG3 isotypes. We tested the capacity of immune complexes prepared with copper-oxidized human LDL and affinity chromatography-purified human OxLDL antibodies [OxLDL-immune complexes (ICs)] to activate complement and to induce cytokine release by MonoMac 6 (MM6) cells and by primary human macrophages. The levels of C4d and C3a were significantly higher in human serum incubated with OxLDL-ICs than after incubation with OxLDL or OxLDL antibody, indicating complement activation by the classical pathway. MM6 cells and primary human macrophages were incubated with OxLDL-ICs, with or without prior conditioning with interferon-γ. After 18 h of incubation, both MM6 cells and primary human macrophages released significantly higher levels of proinflammatory cytokines after incubation with OxLDL-ICs than after incubation with OxLDL or with OxLDL antibody, both in primed and unprimed cells. OxLDL-ICs were more potent activators of MM6 cells than keyhole limpet hemocyanin-ICs. Blocking Fc gamma receptor I (FcγRI) with monomeric IgG1 significantly depressed the response of MM6 cells to OxLDL-ICs. In conclusion, human OxLDL-ICs have proinflammatory properties, as reflected by their capacity to activate the classical pathway of complement and to induce proinflammatory cytokine release from MM6 cells and primary human macrophages.—**Saad, A. F., G. Virella, C. Chassereau, R. J. Boackle, and M. F. Lopes-Virella. **OxLDL immune complexes activate complement and induce cytokine production by MonoMac 6 cells and human macrophages. *J. Lipid Res.* 2006.** 47: **1975–1983.**

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Atherosclerosis is considered the pathologic end point of chronic inflammation of large vessels, triggered by multiple insults and mediated by complex mechanisms, including the activation of immune responses (1, 2). The atheromatous lesions contain modified LDL and corresponding antibodies (3, 4), abundant inflammatory cells such as T-helper-lymphocytes, B-lymphocytes, monocytes/macrophages, and foam cells (cholesteryl ester-rich macrophages). We have described in detail the characteristics of purified antibodies reactive with modified LDL (5–7), the immunogenic modifications of human LDL detected in immune complexes (ICs) isolated from human sera, and the reactivity of IgG fractions isolated from ICs (8). By all criteria, it is possible to establish that oxidized low density lipoprotein (OxLDL) is immunogenic and that it induces an autoimmune response in humans. Isolated human anti-OxLDL antibodies are predominantly of the IgG isotype, subclasses IgG1 and IgG3 (5, 6), and react with malondialdehyde-lysine epitopes found in OxLDL (5, 6) as well as with myeloperoxidase-modified LDL (9).

The fact that OxLDL antibodies are largely of the IgG1 and IgG3 subclasses is a strong indicator of their proinflammatory potential (2). Such antibodies should be able to activate the complement system through the classical pathway and should be able to promote phagocytosis by cells expressing Fcγ receptors (10). Reports from several groups suggesting that complement activation takes place in the atherosclerotic plaque (11, 12) increased our interest in investigating the complement-fixing capacity of OxLDL-ICs, and this became possible as we scaled up our protocol for the isolation of human OxLDL antibodies.

The capacity of OxLDL antibodies to promote the ingestion of OxLDL-ICs by phagocytic cells had been demonstrated by experiments showing that OxLDL-ICs engaged Fcγ receptors of human monocyte-derived mac-

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rophages and of various macrophage-like cell lines such as THP-1 and U-937, leading to intracellular accumulation of cholesteryl esters and to foam cell transformation (13–15). However, the data obtained for cell activation and cytokine secretion when the macrophage cell lines mentioned above were incubated with OxLDL-ICs lacked consistency. This was especially the case in studies carried out with some cell lines, such as THP-1 cells, which need previous incubation with PMA to acquire macrophage-like characteristics. Given the fact that PMA and other phorbol esters are potent nonspecific cell activators, it was not surprising that the baselines in our macrophage experiments were widely variable. For this reason, we performed this study using the MonoMac 6 (MM6) macrophage-like cell line, which more closely mimics the physiologic functions of human monocyte-derived macrophages (16), including vigorous cytokine release after stimulation. In addition, we carried out experiments with primary human macrophages to confirm the relevance of our findings with MM6 cells.

METHODS

Human sera

Human antibodies to OxLDL were purified from sera of patients with type 1 diabetes mellitus as reported in previous publications, using the same patient population (7, 13). Human LDL and antibodies to keyhole limpet hemocyanin (KLH) were obtained from healthy volunteers. Informed consent as approved by the institutional review board for human research of every center involved in sample collection was obtained from each subject included in this study.

Preparation of human OxLDL

Oxidized LDL was prepared by incubation of freshly isolated human LDL at 1,500 mg/l with $CuCl_2$ at a final concentration of 40 μmol/l, as described previously (17). The degree of oxidation was measured by fluorescence emission at 430 nm during a total of 12–18 h. Only preparations reaching an emission fluorescence value of 1.1 units or more during that time frame were used, because such preparations have been shown to react optimally with human OxLDL antibodies (17) and to best absorb purified OxLDL antibodies (6).

Isolation of OxLDL antibodies from human serum

Human OxLDL antibodies were isolated by a two-step protocol. First, IgG was isolated from whole serum by affinity chromatography using immobilized protein G (Protein G-Sepharose 4 Fast Flow; Amersham-Pharmacia Biotech, Piscataway, NJ) according to the protocol provided by the manufacturer. After dialysis against 0.01 mol/l $NaHCO_3$ buffer, pH 8.3, the IgG preparations were fractionated by affinity chromatography in Sepharose-linked OxLDL, as published previously (5, 6). Isolated OxLDL antibodies were passed through a 0.2 μm filter (Nalgene), γ-irradiated to ensure sterility, and centrifuged at 90,000 *g* for 45 min to eliminate aggregates. In addition, the antibodies were screened for endotoxin using the Etoxate kit (Sigma), which is sensitive to levels of 0.005–0.01 ng/ml.

Preparation of OxLDL-ICs

Human OxLDL-ICs were prepared as described by Atchley et al. (13). In this study, the optimal proportions of OxLDL and OxLDL antibody for preparation of insoluble ICs was 150 μg/500 μg (w/w). The precipitates were resuspended and the concentrations of total protein and OxLDL were measured. The concentration of OxLDL was found to vary between 33% and 50% of the total protein concentration. The sterility of resuspended OxLDL-ICs was ensured by γ-irradiation before addition to cell cultures.

Preparation of KLH-ICs

KLH-ICs were prepared with the IgG fraction from anti-KLH human serum obtained by affinity chromatography on immobilized protein G (Protein G-Sepharose 4 Fast Flow; Amersham-Pharmacia Biotech) according to the protocol provided by the manufacturer. The dissociation constant (K_d) of the KLH antibody, calculated by enzymoimmunoassay (5, 18), was 7.6 mol/l × E^{-10}. A precipitin curve indicated that the optimal proportions for precipitation were 200 μg of the KLH antibody IgG fraction and 250 μg of endotoxin-free KLH (Calbiochem). The precipitation process was enhanced by adding 4% polyethylene glycol 6000 to the antigen-antibody mixture. Antibody sterility was ensured by filtration (0.2 μm; Nalgene) and γ-irradiation. KLH-ICs were also γ-irradiated before addition to cell cultures.

C4d and C3a determination and quantification

For the complement activation assay, 40 μg of OxLDL-ICs were resuspended in 1 ml of freshly collected human serum diluted 1:5 with Gelatin-Veronal buffer solution (Sigma-Aldrich) and incubated for 60 min at 37°C. The optimal amount of OxLDL-ICs to be used in these experiments was determined by testing one LDL-IC preparation at different concentrations. Complement fragments C4d and C3a were quantified by enzymoimmunoassay using reagents obtained from Quidel (San Diego, CA). Test samples were first centrifuged at 500 *g*, and the supernatants were harvested and diluted in Quidel's sample diluent to a final dilution of 1:160 for the assay. Each sample was assayed in triplicate. The enzymoimmunoassay data were processed using SOFTmax™ PRO software. Controls consisted of diluted human serum alone, diluted human serum spiked with 15 μg of OxLDL, 25 μg of human OxLDL antibody, and 40 μg of heat-aggregated human IgG [prepared by heating 5 mg of purified human IgG (Pierce Endogen) for 45–60 min at 60°C].

Isolation of peripheral blood monocytes

Peripheral blood monocytes were isolated using the method described by Danciger et al. (19).

Differentiation of monocytes into macrophages

For differentiation into macrophages, the isolated monocytes were counted and resuspended at 1.0×10^6/ml in Iscoves' modified Dulbecco's medium supplemented with 10% whole human serum instead of fetal calf serum, plated on 24-well plates (Falcon), and incubated in humidified 5% CO_2 at 37°C. On the second day, nonadherent cells were removed and fresh medium was added. Medium was replaced a second time on day 6.

MM6 culture conditions

MM6 cells, a human macrophage-like cell line (16), were cultured in a 5% CO_2 humidified atmosphere in RPMI 1640 medium with 2 mM L-glutamine supplemented with 10% fetal calf serum depleted of apolipoprotein B-containing lipoproteins

by ultracentrifugation, 2% nonessential amino acids, 1% oxaloacetate, pyruvate, bovine insulin medium (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich), according to instructions from the American Type Culture Collection. Cells were maintained at a density of 0.3–1.0 $\times$ 10^6 cells/ml, and the medium was changed every 2–3 days. Before use, all media and reagents were screened for endotoxin using the E-Toxate Limulus detection kit (Sigma-Aldrich).

Activation of MM6 and human macrophages with OxLDL-ICs and cytokine assay in media harvested from cell cultures

Experiments were carried out with cells seeded at a density of 1 $\times$ 10^6 cells/ml (0.5 $\times$ 10^6 cells/well) on a 24-well plate. The effects of OxLDL-ICs and controls were studied in interferon-primed and unprimed cultures. Interferon-γ was added at a concentration of 100 ng/ml to prime cultures 24 h before treating the cells with OxLDL-ICs. The concentration of human OxLDL-ICs used in these experiments was 40 μg/ml, which was determined to be optimal after testing OxLDL-IC concentrations from 20 to 75 μg/ml. This concentration is one-tenth of the average concentration of LDL-ICs in 1 ml of serum, as determined in 20 patients with type 1 diabetes. The LDL-IC concentrations in those samples were calculated by adding the amount of antigen and antibody assayed after fractionation of polyethylene glycol-precipitated ICs, as described previously (8). As controls, cells were incubated in identical conditions with buffer alone, OxLDL (15 μg/ml), and human OxLDL antibody (25 μg/ml). Final volumes were adjusted with PBS, pH 7.4. After overnight incubation, the cell supernatants were collected and cytokine quantification was done the same day.

Concentrations of interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor (TNF) were determined by SearchLight multiplex analysis (Pierce Endogen), which uses prespotted plates with up to 10 capture antibodies per well. The entire plate is then imaged to capture the chemiluminescent signal generated at each spot. This assay uses SearchLight CCD Imaging and ArrayVision™ software to calculate concentrations (pg/ml) for unknowns from standard curves. For all cytokine assays, the average intra-assay coefficient of variation (CV) was <5% and the interassay CV was <10%.

To determine whether FcγRI or FcγRII engagement was responsible for the activation of MM6 cells by OxLDL-ICs, we incubated primed MM6 cells with monoclonal IgG1 and IgG2 proteins, previously centrifuged at 100,000 *g* for 45 min to eliminate aggregates, and with CD32 clone FLI8.26 (Stemcell Technologies) of the IgG2b isotype. The blocking reagents were added at concentrations of 5, 10, and 20 μg/ml, and the cells were incubated for 45 min at 4°C. At the end of this preincubation, OxLDL-ICs (20 μg/ml) were added to the MM6 cells. Each experimental point was run in duplicate.

In these experiments, only TNF was measured, in triplicate, using the Quantikine ELISA assay (R&D Systems). The interassay CV of the assay was 5.2%

Statistical analysis

Data were analyzed using InStat (GraphPad, San Diego, CA).The Mann-Whitney test was used to evaluate differences between two treatments. One-way ANOVA was used to compare multiple treatments. Because of the marked differences in cytokine levels obtained with the different treatments, logarithmic transformation of the data was performed, and the Tukey-Kramer multiple-comparison posttest was used to assess differences between treatments. All results, unless indicated otherwise, are expressed as means ± SD.

RESULTS

OxLDL-ICs activate the complement system

As shown in **Fig. 1A**, the concentration of C4d fragments was significantly higher in serum samples incubated with heat-aggregated γ-globulin (4,062 ± 500.1 ng/ml) and OxLDL-ICs (2,140 ± 173.5 ng/ml) than that in serum controls (577.0 ± 52.57 ng/ml; $P = 0.001$) incubated in identical conditions. The concentrations of C4d in serum samples incubated with OxLDL or anti-OxLDL antibody alone (501.3 ± 100.7 and 798 ± 72.57, respectively), in concentrations calculated to reflect the corresponding amounts in OxLDL-ICs, were not significantly different

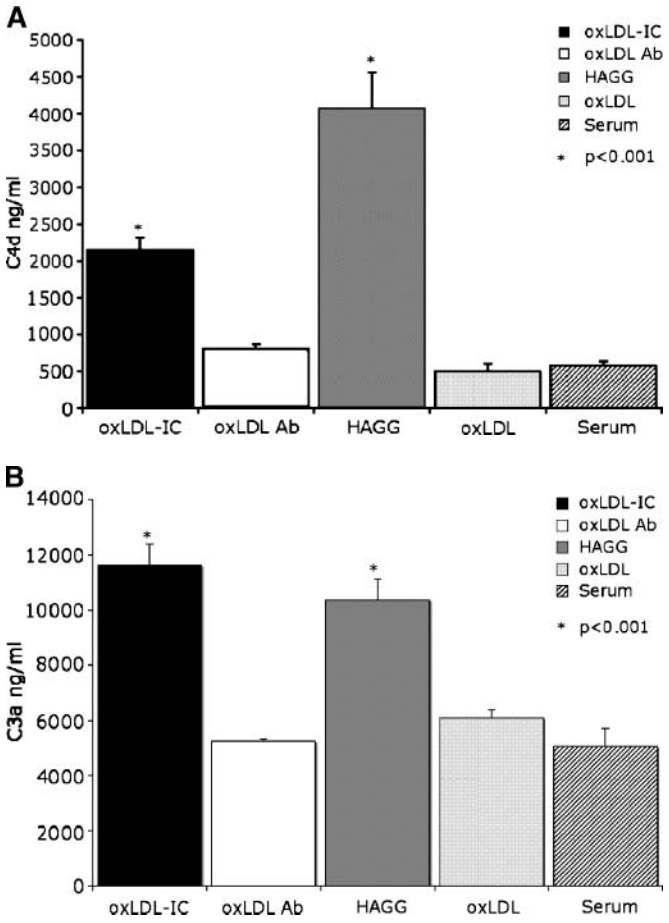

Fig. 1. Study of the complement system activation by oxidized low density lipoprotein immune complexes (OxLDL-ICs). The levels of C4d and C3a fragments (A, B, respectively) were measured in serum samples incubated with OxLDL-ICs (40 μg/ml), human OxLDL antibody alone (OxLDL Ab; 25 μg/ml), heat-aggregated γ-globulin (HAGG; 40 μg/ml), and human oxidized LDL (OxLDL; 15 μg/ml) as well as in a blank sample of the same serum diluted and processed similarly (Serum). Seven different OxLDL antibodies and corresponding OxLDL-ICs were used in this study. The data are given as means ± SD for each complement fragment. Comparison of different treatments by nonparametric ANOVA (Kruskal-Wallis test) showed a significant difference between treatments ($P < 0.0001$). *P* values correspond to the comparison between the levels measured in serum incubated with OxLDL-ICs and those measured in serum alone (background) calculated by the Mann-Whitney test.

from those measured in the serum control (577.0 ± 52.57 ng/ml; $P > 0.05$). The effects of OxLDL-ICs on C3 are shown in Fig. 1B. The concentrations of C3a fragments were higher when serum was incubated with heat-aggregated γ-globulin (10,363 ± 771 ng/ml; $P = 0.001$) or OxLDL-ICs (11,626 ± 760.4 ng/ml; $P = 0.001$) than in serum controls (5,050 ± 669.6 ng/ml). The concentrations of C3a measured in serum samples incubated with OxLDL (6,103 ± 291 ng/ml) or anti-OxLDL antibody (5,251 ± 100.6 ng/ml) alone were not significantly different from those measured in serum controls (5,050 ± 669.6 ng/ml; $P > 0.05$).

OxLDL-ICs induce the release of proinflammatory cytokines by MM6 cells

The activation of MM6 cells was assessed by measuring cytokine levels in the culture medium. We chose cytokines known to be released by activated macrophages and MM6 cells, mostly with proinflammatory properties, but we also measured IL-10, which is considered an anti-inflammatory cytokine. OxLDL, medium alone (untreated), and the IgG fraction of the anti-OxLDL antibody used to prepare the OxLDL-IC were used as controls. Using unprimed MM6 cells, OxLDL-IC induced a significant release of cytokines (IL-1β, IL-12p70, IL-6, TNF, and IL-10) compared with OxLDL or OxLDL antibody alone added to the cultures in concentrations analogous to those present in OxLDL-ICs (**Fig. 2**) ($P < 0.001$). The experiment was repeated in the same exact conditions using MM6 cells primed by preincubation with interferon-γ for 24 h before adding OxLDL-ICs or controls to the cells (**Fig. 3**). In general, interferon priming resulted in the release of significantly higher levels of cytokines by MM6 cells incubated with OxLDL-ICs, with the exception of IL-10 and IL-12p70 (**Fig. 4**).

To investigate the reproducibility of these findings, we performed four additional experiments with primed MM6 cells using different preparations of OxLDL-ICs obtained with OxLDL antibodies isolated from six different individuals (**Fig. 5**). The data obtained in this second set of experiments were very similar to our initial data. All prepaparations of OxLDL-ICs induced significant release of IL-1β, IL-6, IL-10, IL-12p70, and TNF compared with controls of OxLDL or OxLDL antibody alone. Similar results were obtained measuring IL-8. In unprimed cells, the levels in the supernatants of MM6 cells incubated with OxLDL-ICs (18,111 ± 698 ng/ml) were significantly higher ($P < 0.001$) than those measured in the supernatants of MM6 cells incubated with OxLDL (3,137 ± 1,204 ng/ml) or OxLDL antibody (2,653 ± 1,093 ng/ml). The same was observed in primed cells. The IL-8 levels released by MM6 cells incubated with OxLDL-ICs (251,579 ± 116,676 ng/ml) were significantly higher ($P < 0.001$) than those measured in the supernatants of MM6 cells incubated with OxLDL (2,392 ± 323 ng/ml) or OxLDL antibody (20,053 ± 4,396 ng/ml).

OxLDL-ICs induce the release of proinflammatory cytokines by human macrophages

The effect of OxLDL-ICs in human monocyte-derived macrophages was tested using identical conditions to those described for the experiments carried out with MM6 cells. In unprimed human monocyte-derived macrophages, OxLDL-ICs induced a significant release of cytokines (IL-1β, IL-6, IL-10, IL-12p70, and TNF) compared with OxLDL or OxLDL antibody ($P < 0.001$). The experiment was also repeated in the same exact conditions using macrophages primed by preincubation with interferon-γ for 24 h before adding treatments or controls to the cells. Again, priming the cells enhanced the release of cytokines by macrophages incubated with OxLDL-ICs. The cytokine levels measured in primed macrophages exposed to OxLDL-ICs were significantly higher than those measured in the supernatants of macrophages incubated with OxLDL antibody, OxLDL, or medium (**Fig. 6**).

The activation of MM6 cells by human OxLDL-ICs is mediated by engagement of the FcγRI

An experiment was carried out using MM6 cells stimulated with a low dose (20 μg/ml) of OxLDL-ICs after

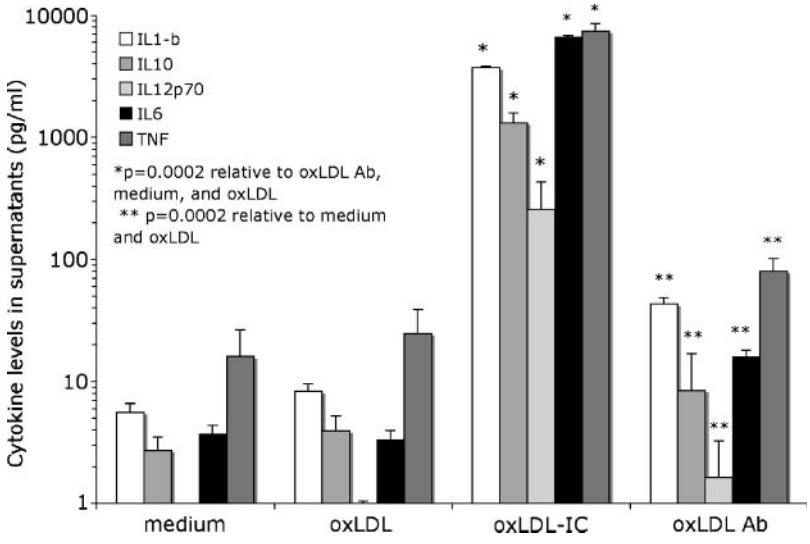

Fig. 2. Results of cytokine assay on the supernatants of unprimed MonoMac 6 (MM6) cells. MM6 cells (1×10^6/ml) were incubated for 18 h with OxLDL (15 μg/ml), OxLDL-ICs (40 μg/ml), or human OxLDL antibody (OxLDL Ab; 25 μg/ml). Four different antibodies were used in this study. After incubation, the culture medium was collected and cytokine levels were measured (see Methods). The data are given as means ± SD for each cytokine, measured in quadruplicate. Comparison of different treatments by one-way ANOVA after logarithmic transformation of the data showed a significant difference between treatments ($P < 0.0001$). *P* values correspond to the comparison between the levels measured in serum incubated with OxLDL-ICs and those measured in serum alone (background) using the Tukey-Kramer multiple-comparisons test. IL-1β, interleukin-1β; TNF, tumor necrosis factor.

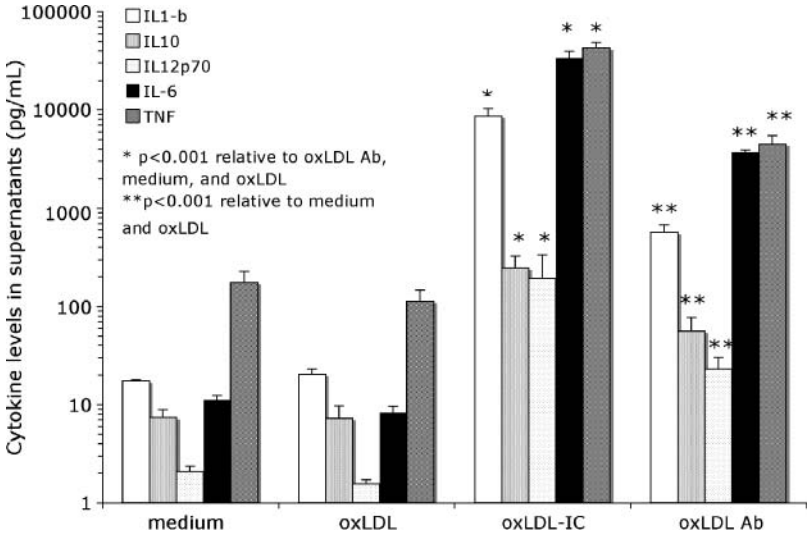

Fig. 3. Results of cytokine assay on the supernatants of interferon-γ-primed MM6 cells. MM6 cells (1×10^6/ml) were primed by incubation with interferon-γ (100 ng/ml) for 24 h. After priming, the cells were incubated for 18 h with OxLDL (15 μg/ml), OxLDL-ICs (40 μg/ml), or human OxLDL antibody (OxLDL Ab; 25 μg/ml). Four different antibodies were used in this study. After incubation, the culture medium was collected and cytokine levels were measured (see Methods). The data are given as means ± SD for each cytokine, measured in quadruplicate. Comparison of different treatments by one-way ANOVA after logarithmic transformation of the data showed a significant difference between treatments ($P < 0.0001$). *P* values correspond to the comparison between the levels measured in serum incubated with OxLDL-ICs and those measured in serum alone (background) using the Tukey-Kramer multiple-comparisons test.

preincubation with monoclonal human IgG1 to block FcγRI and a CD32 monoclonal antibody to block FcγRII. The levels of TNF were measured in the supernatants, as the end point for MM6 activation. Human IgG1 at concentrations of 10 and 20 μg/ml significantly reduced TNF release by MM6 cells stimulated with OxLDL-ICs by 39% and 60%, respectively ($P < 0.001$). In contrast, neither human IgG2 (a specificity control for IgG1) nor CD32 monoclonal antibody blocked the effect of OxLDL-ICs.

Release of cytokines by MM6 cells incubated with purified OxLDL antibodies

Although the difference in the levels of proinflammatory cytokines in both MM6 and human monocyte-derived macrophage cultures incubated with OxLDL-ICs was highly significant, there is no question that, in contrast with what happens in human monocyte-derived macrophages, in unprimed and interferon-γ-primed MM6 cells OxLDL antibody alone is able to stimulate cytokine release (Figs. 3, 4). Three possibilities were considered: the presence of IgG aggregates in the antibody preparations, the formation of LDL-ICs by cross-reactivity of human OxLDL antibody with bovine LDL in the medium, and contamination with endotoxin. Neither antibody centrifugation at 90,000 *g* nor the use of fetal calf serum depleted of apolipoprotein B-containing lipoproteins in the culture medium prevented the activation of MM6 cells. To determine whether endotoxin could be responsible for our observations, we measured endotoxin levels in our antibody preparations and detected levels of <0.02 ng/ml, barely above the detection limit (0.005–0.01 ng/ml). However, given the high sensitivity of MM6 cells to endotoxin (20), we studied the effect of a 4-fold higher concentration of endotoxin (0.08 ng/ml). As shown in **Table 1**, even at this relatively high concentration, endotoxin is a weaker stimulus than OxLDL antibody.

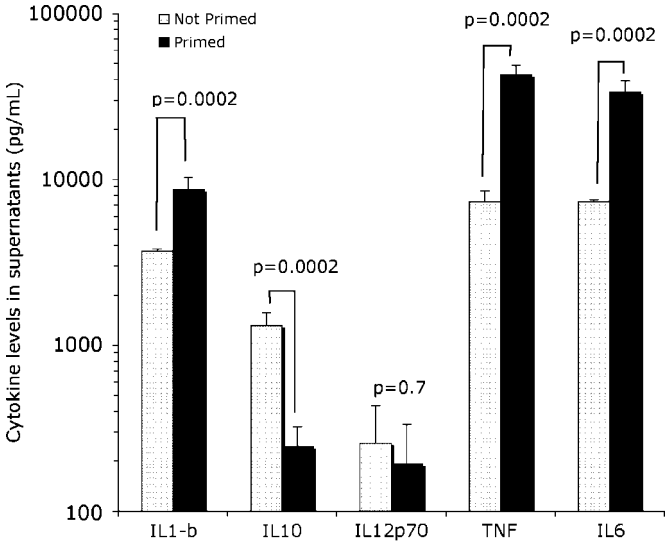

Fig. 4. Comparison of the cytokine levels measured in primed and unprimed MM6 cells incubated with OxLDL-ICs (see Figs. 2, 3 for details). Four different antibodies were used in this study. The data are given as means ± SD for each cytokine, measured in quadruplicate. *P* values were calculated using the Mann-Whitney test.

The experiments carried out with human monocyte-derived macrophages failed to show significant differences between unprimed cells incubated in medium and cells incubated with OxLDL antibody. The levels of IL-1β were the only ones significantly higher in interferon-γ-primed human monocyte-derived macrophages incubated in the presence of OxLDL antibody than in primed human primary macrophages incubated in medium ($P < 0.01$).

A final issue that we investigated was the comparison of OxLDL-ICs with ICs prepared with a protein with molecular weight close to that of apolipoprotein B to evaluate their relative potency as MM6 cell activators, avoiding differences that could be related to antigen size. For this purpose, we used anti-KLH-ICs prepared with human antibody. As shown in **Fig. 7**, both types of ICs activate MM6 cells, but OxLDL-ICs are more potent inducers of cytokine release.

DISCUSSION

The definition of the proinflammatory mechanisms involved in atherosclerosis is critical for the development

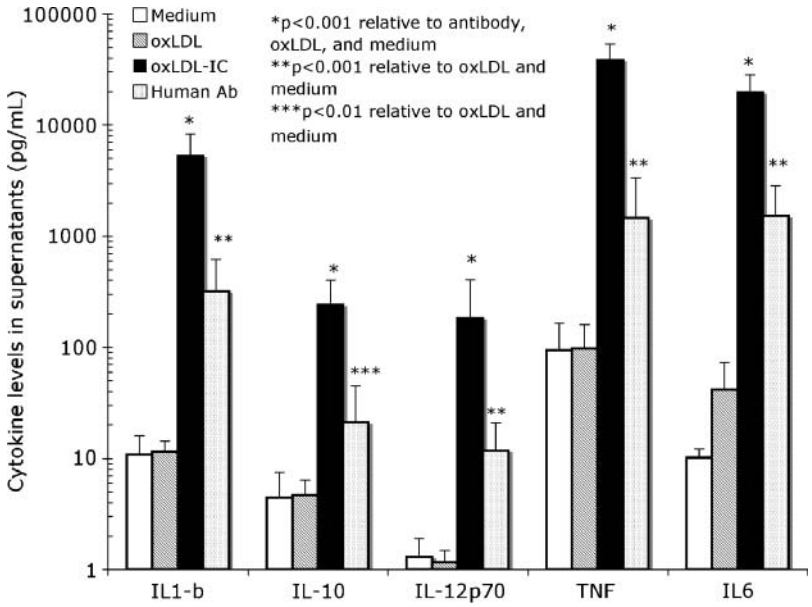

Fig. 5. Summary of data from four experiments in which primed MM6 cells were incubated with OxLDL-ICs prepared using six different human antibodies. MM6 cells (1×10^6/ml) were primed by incubation with interferon-γ (100 ng/ml) for 24 h. After priming, the cells were incubated for 18 h with OxLDL (15 μg/ml), OxLDL-ICs (40 μg/ml), or human OxLDL antibody (OxLDL Ab; 25 μg/ml). The data are given as means ± SD for each cytokine, measured in quadruplicate. Comparison of different treatments by one-way ANOVA after logarithmic transformation of the data showed a significant difference between treatments ($P < 0.0001$). *P* values correspond to the comparison between the levels measured in serum incubated with OxLDL-ICs and those measured in serum alone (background) using the Tukey-Kramer multiple-comparisons test.

of new diagnostic, preventive, and therapeutic approaches. Although there is a general consensus supporting the involvement of immunological mechanisms in the pathogenesis of atherosclerosis (2, 21, 22), there are real differences of opinion about the significance of humoral immunity mechanisms, particularly of the autoimmune response elicited by modified lipoproteins (23–26). Most of the controversy centers on whether such antibodies have proinflammatory or protective properties and it is a result of data obtained in animal models (27–30). However, none of the animal models for atherosclerosis can be considered adequate for the understanding of the role of immune mechanisms, because there are significant species-related differences in lipid metabolism and immune responsiveness. Furthermore, the conclusions reached in some of the studies can also be questioned on the basis of poor experimental design (26). Conflicting data have also been reported in clinical studies: most support a pathogenic effect of modified lipoprotein antibodies, whereas others suggest a protective role (2). The vast majority of clinical studies have been based on the assay of circulating antibodies to different forms of modified LDL. The methodology is flawed and difficult to reproduce (31), and for these reasons, it is likely to be a significant factor leading to conflicting results obtained by different groups.

Knowing that OxLDL antibodies are predominantly IgGs of subclasses 1 and 3 (5, 6, 8), the predicted result of our complement activation experiments was that the classical pathway should be activated by OxLDL-ICs. To prove this point, we incubated human OxLDL-ICs with normal human serum and measured the levels of C3a and C4d. C3 is fragmented whenever complement is activated, by any of the known pathways, but C4 is fragmented only when the classical pathway is activated (32). Thus, our finding of increased levels of both C3a and C4d proves that OxLDL-ICs activate the complement system through the classical pathway. This finding is specially relevant if we consider that complement fragments and immunoglobulins colocalize

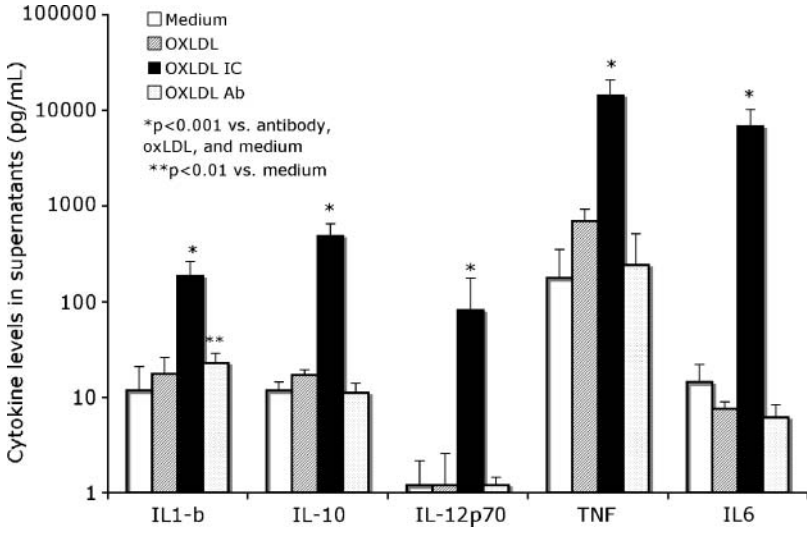

Fig. 6. Summary of data from experiments in which primed human macrophages were incubated with OxLDL-ICs prepared using four different human antibodies. Human macrophages (1×10^6/ml) were primed by incubation with interferon-γ (100 ng/ml) for 24 h. After priming, the cells were incubated for 18 h with OxLDL (15 μg/ml), OxLDL-ICs (40 μg/ml), or human OxLDL antibody (OxLDL Ab; 25 μg/ml). After incubation, the culture medium was collected and cytokine levels were measured (see Methods). The data are given as means ± SD for each cytokine, measured in quadruplicate. Comparison of different treatments by one-way ANOVA after logarithmic transformation of the data showed a significant difference between treatments ($P < 0.0001$). *P* values correspond to the comparison between the levels measured in serum incubated with OxLDL-ICs and those measured in serum alone (background) using the Tukey-Kramer multiple-comparisons test.

TABLE 1. Comparative study of the response of MM6 cells to endotoxin and to human OxLDL antibody

Cytokine	Endotoxin[a] (0.08 ng/ml)	Human OxLDL Antibody[b] (25 μg/ml)	OxLDL-ICs[c] (40 μg/ml)
IL-1α	28 ± 7	54 ± 6 ($P = 0.03$)[d]	2,605 ± 295
IL-1β	93 ± 12	234 ± 20 ($P < 0.0001$)[d]	6,569 ± 1327
IL-6	1,135 ± 230	2,368 ± 275 ($P = 0.01$)[d]	25,236 ± 5098
IL-10	14 ± 3	26 ± 3 ($P = 0.004$)[d]	91 ± 13
IL-12p70	6 ± 1	8 ± 1 (NS)[d]	30 ± 7
Tumor necrosis factor	550 ± 97	1,481 ± 163 ($P < 0.0001$)[d]	36,451 ± 7328

IC, immune complex; IL, interleukin; MM6, MonoMac 6; OxLDL, oxidized low density lipoprotein.

[a] Levels of endotoxin detected in five different OxLDL antibody preparations were <0.02 ng/ml.

[b] Calculated to correspond to the average concentration of OxLDL antibody in 40 μg of OxLDL-ICs.

[c] Used as a positive control. The differences between cytokine levels after incubation with OxLDL antibody and OxLDL-ICs were of the same magnitude observed in previous experiments (see Fig. 5).

[d] *P* values correspond to the comparison between cytokine levels in MM6 cultures stimulated with endotoxin versus OxLDL antibody by the Mann-Whitney test.

in atherosclerotic lesions (33). Therefore, OxLDL-ICs are likely to be a major factor leading to complement activation in the atheromatous lesion. Once the activating cascade is triggered, anaphylotoxins such as C3a and C5a will be generated, and they can contribute to the acceleration of the inflammatory reaction through their chemotactic properties and by inducing the expression of cell adhesion molecules on endothelial cells (33). The generation of terminal complement components has also been documented in the atheromatous lesions, and there is experimental evidence suggesting that these components are equally proinflammatory through the activation of endothelial cells, leading to the release of chemokines and other mediators and inducing the proliferation of vascular smooth muscle cells (33, 34).

Equally consistent with the structure of OxLDL antibodies was the unequivocal evidence of the activation of MM6 cells and human monocyte-derived macrophages by OxLDL-ICs prepared with human OxLDL and human antibodies, as reflected by the release of high concentrations of proinflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12p70, and TNF. All of these have been demonstrated in atheromatous lesions, where they play key roles in the initiation and perpetuation of the vascular inflammatory process (2).

The enhanced release of cytokines by MM6 cells and human monocyte-derived macrophages after priming with interferon-γ is quite interesting. Activated $CD4^+$ T-lymphocytes and macrophages are the predominant cell populations identifiable in atheromatous plaques (35, 36).

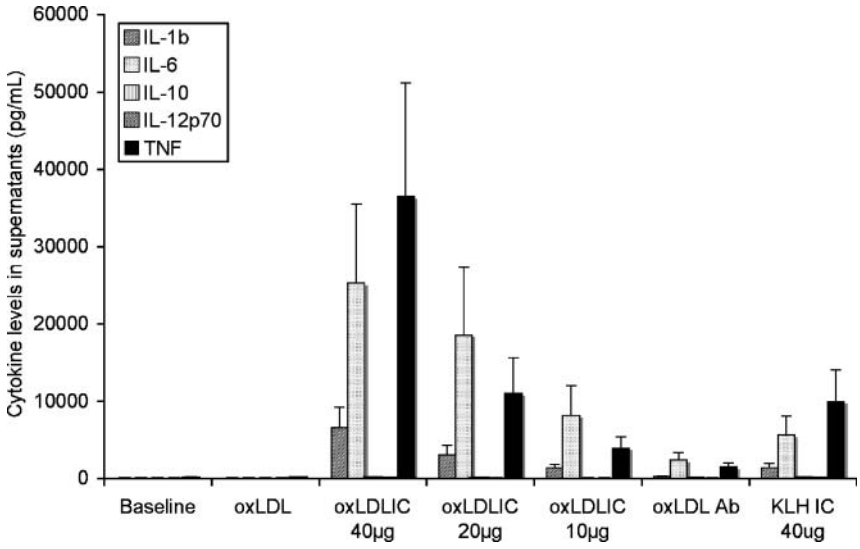

Fig. 7. Comparison of the effects of OxLDL-ICs and keyhole limpet hemocyanin (KLH)-ICs on primed MM6 cells. MM6 cells (1×10^6/ml) were primed by incubation with interferon-γ (100 ng/ml) for 24 h. After priming, the cells were incubated for 18 h with three different concentrations of OxLDL-ICs prepared with two different OxLDL antibodies with KLH-ICs at a concentration identical to the higher concentration of OxLDL-ICs. As controls, we incubated the cells with medium alone, OxLDL (20 μg/ml), and OxLDL antibody (OxLDL Ab; 20 μg/ml). The data are given as means ± SD for each cytokine, measured in quadruplicate. Comparison of different treatments by one-way ANOVA after logarithmic transformation of the data showed a significant difference between treatments ($P < 0.0001$). *P* values correspond to the comparison between the levels measured in serum incubated with OxLDL-ICs and those measured in serum alone (background) using the Tukey-Kramer multiple-comparisons test.

Those activated $CD4^+$ T-cells do not fit neatly into the Th1-Th2 pattern, but they release predominantly interferon-γ, a cytokine that is believed to play a key role in the development of atherosclerosis (36). This implies that cell-mediated and humoral mechanisms may synergize in the atheromatous plaque to enhance the inflammatory reaction. The decreased release of IL-10 by interferon-γ-primed MM6 cells would favor this synergism by reducing the downregulation of inflammatory processes, but it was not observed in the experiments carried out with human monocyte-derived macrophages, so its significance is unclear.

Previous work from our laboratory had shown that FcγRI was the primary receptor involved in the interaction of LDL-ICs with human macrophages and macrophage-like cell lines (15). However, in that study, we used rabbit apolipoprotein B antibodies and human LDL, so it was important to verify that the same would be true for MM6 cell activation using human OxLDL-ICs. Our results show that FcγRI is the primary Fcγ receptor involved, but the inhibition of MM6 activation was not complete (60% with 20 μg/ml IgG1 protein, the highest concentration tested). This raises the possibility that other receptors may be involved in MM6 cell activation, such as scavenger receptors, an issue that will be the subject of future investigations.

We also observed that OxLDL antibody alone induced the release of low levels of cytokines in unprimed cells, levels that were significantly increased in primed MM6 cells ($P < 0.005$). In contrast, neither primed nor unprimed human monocyte-derived macrophages responded to OxLDL antibody alone in the way that MM6 cells did. Only IL-1β showed a marginal increase in supernatants of macrophages incubated with OxLDL relative to those incubated with medium alone. This suggests that the immortalizing mutations of MM6 cells may enhance the sensitivity of FcγRI to either monomeric OxLDL antibody or small concentrations of aggregates that remain or reform after ultracentrifugation of the antibody. Other factors, such as the formation of LDL-ICs by cross-reactive OxLDL antibodies and bovine LDL or contamination of the antibody preparations with endotoxin, were excluded in our experimental protocol.

Also worth noting is the fact that in our experiments OxLDL-ICs appear to be more potent than KLH-ICs. This finding is unexpected for two reasons. First, other groups have not observed differences between the two types of ICs (37). The other is that given the fact that KLH is a heterologous protein, the immune response to KLH is not only very vigorous but leads to the synthesis of high-affinity antibodies (the antibody used in our study had a K_d of $7.6\ \text{mol/l} \times E^{-10}$, compared with an average K_d of $1.02 \pm 1.1\ \text{mol/l} \times E^{-8}$ for our purified OxLDL antibodies) (31). Antibodies of higher affinity should be more efficient in IC formation, leading to the formation of ICs that are more stable and probably larger than those formed with OxLDL. Therefore, one would expect that, in equal concentrations, KLH-ICs would be more effective MM6 cell activators than OxLDL-ICs, rather than the opposite. The higher efficiency of OxLDL-ICs as MM6 cell activators suggests that the antigen moiety (OxLDL), when internalized in the form of an IC, may play a significant role in cell activation.

In conclusion, data generated by structural studies and by in vitro studies of the effects of OxLDL-ICs on inflammatory cells, as well as a growing body of evidence obtained in human studies, suggest that ICs formed by modified LDL and corresponding antibodies play a significant role in atherosclerosis (2). Modified LDL (3, 38, 39), the corresponding antibodies (4), and complement have been demonstrated in atheromatous plaques. Longitudinal clinical studies have demonstrated a significant correlation between the levels of modified LDL-ICs and the development of diabetic nephropathy (13, 40), cardiovascular disease (41, 42), and increased internal carotid intima-media thickness (43). Thus, there is a solid body of evidence supporting a pathogenic role for the humoral autoimmune response to modified lipoproteins in humans.

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