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The Common Vaccine Adjuvant Aluminum Hydroxide Up-Regulates Accessory Properties of Human Monocytes via an Interleukin-4-Dependent Mechanism

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Aluminum adjuvants are widely used in human vaccines based on their ability to enhance antibody production. However, the mechanisms underlying these effects remain unknown. In the present study we assessed the direct in vitro effect of aluminum hydroxide on human peripheral blood monocytes, specifically with regard to its impact on the phenotype and functional properties of this cell population. Our results revealed significant changes in the accessory properties of monocytes following short-term exposure of cultured cells to aluminum hydroxide. Thus, flow cytometry analyses showed an increase in the expression of major histocompatibility complex (MHC) class II, CD40, CD54, CD58, CD83, and CD86 molecules on the monocytes. In addition, many cells in the cultures containing aluminum hydroxide acquired typical dendritic morphology. Increased synthesis of interleukin-4 (IL-4) mRNA, but not gamma interferon mRNA, was also noted after exposure to aluminum hydroxide. The increase in cell surface expression of MHC class II did not occur in the presence of neutralizing IL-4 antibody or in cultures of highly purified monocytes or CD4-depleted mononuclear cells. Our findings suggest that aluminum hydroxide directly stimulates monocytes to produce proinflammatory cytokines activating T cells. Activated Th2 cells release IL-4, which in turn can induce an increase in the expression of MHC class II molecules on monocytes. The increase in the expression of antigen-presenting and costimulatory molecules leads to enhanced accessory functions of monocytes. These properties of aluminum hydroxide observed in vitro may explain its potent in vivo adjuvant effect.

Aluminum compounds have been widely used as human vaccine adjuvants for more than 70 years. It is known that their immunoadjuvant effect is associated with the induction of Th2 responses (3, 4, 16). However, the mechanisms underlying this effect remain unknown. It is believed that aluminum adjuvants form a "depot" at the site of injection from which antigen is released slowly, leading to a prolonged exposure to antigen-presenting cells and lymphocytes (25). It has also been demonstrated that aluminum hydroxide enhances antigen uptake by the antigen-presenting cells in vitro (26). Whether aluminum adjuvants elicit any direct stimulatory effect on cells involved in immune responses remains unknown. Aluminum compounds are themselves not immunogenic, nor do they act as haptens (29).

In the present study we investigated the direct effect of aluminum hydroxide on human monocytes in in vitro cultures of peripheral blood mononuclear cells (PBMC). Exposure to aluminum hydroxide led to a significant activation of the accessory properties of monocytes. Further experiments showed an involvement of interleukin-4 (IL-4) in the increase of cell surface expression of major histocompatibility complex (MHC) class II molecules induced by aluminum hydroxide. These findings indicate that aluminum hydroxide can directly stimulate antigen-presenting cells, which may represent an important mechanism underlying its in vivo immunoadjuvant effect.

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MATERIALS AND METHODS

Cell preparation and culture conditions. PBMC were separated from heparinized whole blood on a Lymphoprep density gradient (Nycomed Pharma AS, Oslo, Norway), washed, and resuspended in complete medium (RPMI 1640 medium containing 1% L-glutamine (Gibco, Glasgow, Scotland) supplemented with 1% nonessential amino acids (Gibco), 1% sodium pyruvate (Gibco), 5×10^{-5} M β-mercaptoethanol, and 50 μg of gentamicin sulfate (Schering-Plough, Stockholm, Sweden) per ml. The complete RPMI was supplemented with fetal calf serum (Gibco catalog no. 10084-168) (RPMI-FCS). The endotoxin content of culture medium was below 1 pg/ml, as determined by the *Limulus* assay.

For preparation of adherent cells, isolated PBMC were plated (10^7 cells/ml) in six-well plates (Nunclon, Roskilde, Denmark) (3 ml/well) in RPMI-FCS. After 2 h at 37°C, nonadherent cells were removed, and the adherent cells were cultured at 37°C in a humidified 5% CO_2 incubator.

Purified $CD14^+$ monocytes and CD4-depleted mononuclear cells were prepared by magnetic sorting using the MiniMACS technique (Miltenyi Biotech GmbH, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. Briefly, 10^8 PBMC were incubated with colloidal magnetic microbeads conjugated to anti-human CD14 or alternatively to anti-human CD4 (150 μl in 600 μl of phosphate-buffered saline [PBS] containing 0.5% bovine serum albumin [BSA] and 2 mM EDTA) for 30 min at 4°C. After a washing with the same buffer, the cells were passed over a column in a strong magnetic field. Cells coated with CD14 microbeads were then eluted from magnetic columns by removal of the magnetic device (positive selection). Before culturing, we examined the percentage of $CD14^+$ cells in these preparations by flow cytometry and used only cell specimens containing more than 98% $CD14^+$ cells. To obtain the CD4-depleted fraction, cells which had passed through the column were collected (negative selection). The number of remaining $CD4^+$ cells in this fraction was <1.5%, as determined by flow cytometry.

For the study of surface expression of different molecules, cultures of whole PBMC or fractionated mononuclear cells were prepared in six-well plates. The cells (2×10^6 cells/ml) were cultured in 3 ml of RPMI–10% FCS with aluminum hydroxide gel (Statens Seruminstitut, Copenhagen, Denmark; endotoxin content, <1 pg/ml) at concentrations of 1, 5, and 50 μg/ml for 24, 48, or 72 h in a humidified atmosphere of 5% CO_2 at 37°C. Parallel sets of wells containing PBMC exposed to medium alone were used as negative controls.

In some experiments PBMC were cultured with human recombinant cytokines: 1,000 U of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leucomax [Schering-Plough]; specific activity, 11.1×10^6 U/mg) per ml and 400 U of IL-4 (Genzyme, Cambridge, Mass.; specific activity, 10^7 U/mg) per ml. Polyclonal goat anti-human IL-4 antibody (R&D Systems Europe, Abingdon, United Kingdom) or species-matched control immunoglobulins (total goat immunoglobulin G [IgG]) (R&D Systems) were introduced at a final concentration of 10 μg/ml at the time of initiation of the culture. In some experiments, monoclonal antibody (MAb) to human gamma interferon (IFN-γ) (R&D Systems) or an isotype-matched control (mouse IgG2a; Sigma, St. Louis, Mo.) was added to the culture at a concentration of 10 μg/ml. After 48 h of culture, all the cells were harvested by gentle scraping with a rubber policeman, washed, counted, and stained with monoclonal antibodies for flow cytometry analysis.

Culturing of human monocytic cell lines THP-1 and Mono Mac 6 was performed in 10-ml culture flasks (Nunclon) as previously described (39, 48). For stimulation with $Al(OH)_3$, cells from a 48-h culture were harvested, washed, and recultured in six-well plates at a concentration of 10^6 cells/ml in RPMI–10% FCS for 48 h with or without 5 μg of $Al(OH)_3$/ml. In some experiments, THP-1 cells were first stimulated with human recombinant IFN-γ (100 U/ml; Boehringer Mannheim, Mannheim, Germany) in 10-ml culture flasks for 24 h, harvested, washed, and recultured to study the effect of $Al(OH)_3$. After the incubation all the cells were harvested, washed, counted, and analyzed by flow cytometry.

MAbs. For direct immunostaining, mouse MAbs against the following human antigens were used: HLA-DR, -DP, and -DQ (fluorescein isothiocyanate [FITC] and phycoerythrin [PE] conjugated) (CR3/43, IgG1 κ; Dako, Glostrup, Denmark), HLA-DR (PE conjugated) (L243, IgG2a; Becton Dickinson, San Jose, Calif.), HLA-DP (FITC conjugated) (HI43, IgG1 κ; PharMingen, San Diego, Calif.), HLA-DQ (FITC conjugated) (SK10, IgG1; Becton Dickinson), HLA-A, -B, and -C (FITC conjugated) (G46-2.6, IgG1 κ; PharMingen), CD3 (PE conjugated) (LeuTM-4, IgG1 κ; Becton Dickinson), CD14 (PE conjugated) (MϕP9, IgG2b κ; Becton Dickinson), CD19 (PE conjugated) (HD37, IgG1 κ; Dako), CD40 (FITC conjugated) (5C3, IgG1 κ; PharMingen), CD54 (PE conjugated) (LB-2, IgG2b; Becton Dickinson), CD58 (PE conjugated) (L306.4, IgG2a; Becton Dickinson), CD83 (PE conjugated) (HB15A, IgG2b; Immunotech, Marseille, France), CD86 (FITC conjugated) (BU63, IgG1; Serotec, Oxford, United Kingdom).

For intracellular staining of CD68, a PE-conjugated mouse anti-human MAb set containing CD68 antibody (clone Y1/82A) and the corresponding isotype control (mouse IgG2b, clone 27-35) (PharMingen) was used.

Immunofluorescent staining and flow cytometry analysis. Cultured cells (2×10^5) were preincubated for 15 min at 4°C with human gamma globulin (Pharmacia and Upjohn, Uppsala, Sweden) at 500 μg/ml to block nonspecific Fc receptor-mediated binding of MAb and then with MAb directly conjugated to FITC or PE for 20 min at 4°C.

The corresponding isotype-matched controls were used for each sample (mouse IgG1-FITC and IgG2a-PE from Becton Dickinson and IgG2b-FITC and IgG2b-PE from PharMingen).

For intracellular staining, cells were consecutively treated with FACS lysing solution and FACS permeabilizing solution (both from Becton Dickinson) prior to addition of either PE-conjugated CD68 MAb or IgG2b-PE (isotype control) according to the manufacturer's instructions.

After two washing steps the cells were subjected to flow cytometry analysis by a FACSort (Becton Dickinson) using CELLQuest software (Becton Dickinson). Individual populations of cells were gated according to their forward- and side-scatter characteristics.

Immunocytochemistry. For immunocytochemistry analyses, PBMC were cultured in two-well chamber slides (Nunc, Naperville, Ill.) at a concentration of 2×10^6 cells/ml in RPMI–10% FCS (1.5 ml/well) in a humidified atmosphere of 5% CO_2 at 37°C. After 48 h of incubation the chambers were gently rinsed with warm PBS and air dried. The upper structure was removed, and the slides were fixed in ice-cold acetone (30 s in 50% acetone followed by 5 min in 100% acetone) and then air dried for 30 min. After this, endogenous peroxidase activity was blocked by incubation of the slides for 20 min at room temperature in a solution of 1 U of glucose oxidase (Sigma) per liter, 10 mM glucose, and 1 mM NaN_3.

For immunostaining the slides were incubated overnight in a humid chamber at 4°C with purified mouse MAb to MHC class II (CR3/43, IgG1 κ; Dako) diluted 1:1,000 in PBS-Tween (0.05% Tween) containing 2% BSA. Slides used as negative controls were incubated with an isotype-matched irrelevant MAb (mouse IgG1; Becton Dickinson).

After three 5-min washings with PBS, the slides were incubated for 1 h with a biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulins (Dako) diluted 1:400 in PBS-Tween–2% BSA–2% normal human serum. Thereafter, the slides were incubated with avidin-conjugated peroxidase (ABC complex; Dako) for 30 min. The peroxidase deposition was revealed using substrate containing amino-ethyl-carbazole (Sigma) and H_2O_2, followed by a light counterstaining with Mayer's hematoxylin.

Analysis of cytokine mRNA expression by reverse transcription-PCR (RT-PCR). Culturing of PBMC for mRNA analysis was performed as described above, except that the cells were harvested after 24 h of incubation either with or without aluminum hydroxide (5 μg/ml) and washed, and the pellet was frozen at −137°C until further analysis.

Total RNA was isolated from the cultured cells with the SV total RNA isolation system (Promega Corporation, Madison, Wis.) according to the manufacturer's instructions.

The yield and purity of isolated RNA was determined by spectrophotometric measurement of absorbance at 260 and 280 nm. RNA was checked for quality on a 3% agarose gel with ethidium bromide (Sigma Chemical Co.) and in the presence of an RNA standard (human HeLa cell total RNA; Clontech Laboratories Inc., Palo Alto, Calif.). If the isolated RNA was found to have distinct 28S and 18S ribosomal bands, it was considered intact and of good quality.

For cDNA synthesis, 2 μg of RNA and random hexamer (Pharmacia, Uppsala, Sweden) were used together with a mixture of deoxynucleoside triphosphates (Pharmacia), RNase inhibitor from placenta (Promega), SuperScript reverse transcriptase (GIBCO BRL Life Technologies Inc., Gaithersburg, Md.), first-strand buffer (GIBCO), and diethyl pyrocarbonate-treated water in a total volume of 30 μl.

For each cytokine, commercially available specific primers and positive controls (Clontech) were used. As an internal control for the amount of RNA used, the housekeeping gene for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Clontech) was simultaneously amplified.

PCR amplification of 2 μl of cDNA was performed in the presence of AmpliTaq Gold DNA polymerase, PCR buffer, 2.0 mM $MgCl_2$ (all from Perkin-Elmer Cetus, Norwalk, Conn.), deoxynucleoside triphosphate mix (Pharmacia), and sterile water in a total volume of 50 μl. The PCR was performed in a Perkin-Elmer Cetus GeneAmp PCR system 9600. The first step was incubation at 95°C for 12 min and then 35 cycles (IL-1α, IL-1β, IL-10, tumor necrosis factor alpha [TNF-α], and transforming growth factor beta [TGF-β]) or 43 cycles (IL-2, IL-4, IL-5, IL-6, IFN-γ, and GM-CSF) of denaturation at 94°C for 45 s, annealing at 65°C for 45 s, and extension at 72°C for 2 min, followed by a final extension for 7 min at 72°C.

Negative controls consisted of samples in which the reverse transcriptase was omitted to verify the absence of contamination by genomic DNA and a reagent control in which cDNA was replaced with sterile distilled H_2O.

The PCR products were tested by submarine electrophoretic separation (GNA-200 gel electrophoresis apparatus; Pharmacia). Ten microliters of sample mixed with 5 μl of gel loading solution (Sigma) was run on a 3% agarose gel (agarose MS-12, Pronadisa; Hispanlab, S.A., Madrid, Spain) containing ethidium bromide (Sigma) and in the presence of the molecular size marker *Hae*III-digested ϕX174 DNA (GIBCO).

Gel images were scanned (StudioScan II/si; Agfa), and densitometric analysis (i.e., quantification of bands in terms of position, intensity, and area, referred to as volume analysis) was performed with IPLab Gel software for Macintosh (BioSystematica, Plymouth, United Kingdom). The results were expressed as a ratio calculated from the "volume" of the amplified cytokine gene product divided by the "volume" of the amplified G3PDH housekeeping gene product in the same sample (densitometry units).

Statistical methods. Statistical evaluation of the data was performed using a paired nonparametric Wilcoxon signed-rank test. Differences were considered significant if the *P* value was below 0.05.

RESULTS

Aluminum hydroxide increased the cell surface expression of MHC class II, costimulatory, and adhesion molecules on monocytes. The direct effect of different concentrations of aluminum hydroxide gel on human PBMC was tested at different culture times. Our choice of concentrations was based on the awareness that concentrations of 3 mg/ml and higher were found to be toxic for macrophages in an in vivo model in guinea pigs (15) and that the usual dose of aluminum adjuvant for human vaccines is around 0.5 mg applied in 0.1 ml of saline (17).

Our preliminary experiments indicated, indeed, that concen-

trations of $Al(OH)_3$ gel in culture medium of 400 μg/ml and higher were toxic (induced >30% dead cells after 48 h of culturing as detected by the trypan blue exclusion test). Therefore, we used concentrations of 1, 5, and 50 μg of $Al(OH)_3$/ml and cultured thecells for 24, 48, and 72 h.

Exposure of the PBMC to $Al(OH)_3$ at 1, 5, and 50 μg/ml induced visible changes in light-scattering properties of the monocytes, as detected by flow cytometry analysis indicating augmentation of both size and granularity of the cells (Fig. 1 and data not shown). In contrast, the light-scattering properties of the cells in the lymphocyte gate remained unchanged during the experiments. The shift in the light-scattering properties of monocytes upon exposure to $Al(OH)_3$ was comparable to that induced by a combination of GM-CSF and IL-4, the cytokines known as potent activators of monocytes and triggers of their maturation to dendritic cells.

In parallel, there were dose-dependent changes in the expression of MHC class II molecules (Fig. 1D). The highest increase of the MHC class II expression was observed after 48 h of cell culture with 5 μg of $Al(OH)_3$/ml. These culture conditions were used throughout for subsequent analyses.

The similar increase in the expression of MHC class II molecules was detected using MAbs directed to both HLA-DR (L243) and HLA-DP (HI43), as well as a MAb recognizing the common epitope for all the MHC class II molecules (CR3/43). The latter MAb reacts with the β-chain of all products of the DP, DQ, and DR subregions. In contrast, the cell expression of HLA-DQ and MHC class I molecules was not affected by incubation with aluminum hydroxide (Fig. 2A).

Since the observed increase of MHC class II expression on monocytes may imply a change in their accessory characteristics, we analyzed the expression of some other surface molecules known to act as costimulatory and adhesion molecules in the process of antigen presentation. Indeed, as shown in Fig. 2B, a significant increase of the intensity of the expression of CD86, CD54, and CD58 occurred upon exposure of the cultured cells to $Al(OH)_3$. The percentage of $CD86^+$ and $CD40^+$ cells within the monocyte gate increased as well (Fig. 2C). Since CD54 and CD58 antigens were present on almost all of the monocytes even in the control culture, there was no further increase in the percentage of positive cells upon addition of aluminum hydroxide (data not shown). Interestingly, an increase in the percentage of $CD40^+$ monocytes brought about by $Al(OH)_3$ was not accompanied by any increase in the mean fluorescence intensity, indicating that the density of the expression of this molecule remained stable (Fig. 2B, C).

CD83 was recently described as a marker for human mature dendritic cells, although the functional significance of this molecule still remains unknown (46, 47). Of interest, the percentage of $CD83^+$ cells in the cell culture exposed for 48 h to $Al(OH)_3$ increased by a factor of 3 (Fig. 2C).

In order to further characterize the phenotypic changes of monocytes cultured in the presence of $Al(OH)_3$, we performed double staining for the CD86 and HLA-DR antigens. The analysis showed that $Al(OH)_3$ triggered a significant increase in the percentage of $CD86^+$ $HLA\text{-}DR^+$ cells within the monocyte gate (Fig. 3A). In addition, the intensity of MHC class II expression on $CD68^+$ cells (intracellular monocyte/dendritic cell marker) significantly increased upon stimulation with $Al(OH)_3$ (Fig. 3B). Of note, the expression of CD14 decreased in cultures stimulated with $Al(OH)_3$ (data not shown).

Overall, the results indicate the changes in the immunophenotype of monocytes (significant increase in the cellular expression of several surface molecules) during short-term exposure to a low concentration (5 μg/ml) of $Al(OH)_3$. Interestingly, neither the percentage of $CD3^+$ $HLA\text{-}DR^+$ lymphocytes (Fig. 3A) nor the mean fluorescence intensity of HLA-DR staining on $CD3^+$ cells (data not shown) was altered upon exposure to $Al(OH)_3$.

Next we addressed the question of whether lymphocytes present in the cultures would contribute to the observed changes in the phenotype of monocytic cells. We cultured isolated adherent mononuclear cells (MNC) rather than whole PBMC and exposed them to $Al(OH)_3$ under the conditions described above. The population of adherent cells contained 60 to 65% $CD14^+$ monocytes, in contrast to whole PBMC, which consisted mainly of lymphocytes and contained only 10 to 15% $CD14^+$ monocytes. In all the experiments the magnitude of increase of MHC class II expression on monocytes was somewhat higher in the cultures of adherent cells than in the cultures of whole PBMC (2.1- ± 0.1-fold increase, compared to 1.6- ± 0.1-fold, respectively).

Monocytes exposed to $Al(OH)_3$ have a dendritic appearance. The results of the flow cytometry analyses indicated that upon short exposure of cultured PBMC to $Al(OH)_3$, monocytes developed a phenotype which was very similar to those described for cultured dendritic cells undergoing the process of maturation (MHC class II^{high}, $CD86^{high}$, $CD83^+$, and $CD14^-$ cells) (34, 36, 46).

To study the morphologic properties of the cultured cells exposed to $Al(OH)_3$, we performed immunoperoxidase staining with MAbs specific for MHC class II. Immunocytochemistry analysis revealed that the morphology of the stained cells was indeed similar to that of dendritic cells (Fig. 4A). These cells were big, had an irregular shape, had pleomorphic cytoplasmic processes, and showed high expression of MHC class II molecules both intracellularly and on the cell surface. However, not all the cells in the culture showed the dendritic morphology. Some of them looked like macrophages and some looked like monocytes (round, smaller). In contrast, cells with dendritic morphology were only rarely detected in control cultures (Fig. 4B).

Aluminum hydroxide-triggered mRNA expression of monocyte-derived and Th2 type cytokines. To study the effect of aluminum hydroxide on cytokine induction, we performed RT-PCR analysis of mRNA gene expression for monocyte and T-cell cytokines. RT-PCR analysis showed that exposure of PBMC to aluminum hydroxide induced an increased expression of monocyte-derived cytokines (IL-1α, IL-1β, and TNF) and of Th2 type cytokines (IL-4 and IL-6) (Table 1). On the other hand, there was no induction of the expression of Th1 type cytokines IFN-γ, IL-2, and GM-CSF. We could not see any significant increase in synthesis of mRNA for IL-5, TGF-β, or IL-10 either (Table 1).

Aluminum hydroxide-induced increase in the expression of MHC class II was abolished by neutralization of IL-4. Cytokines known to contribute to the cell surface expression of MHC class II on monocytes are IFN-γ, IL-4, and GM-CSF (6, 12, 13). As shown above, an increase in synthesis of IL-4

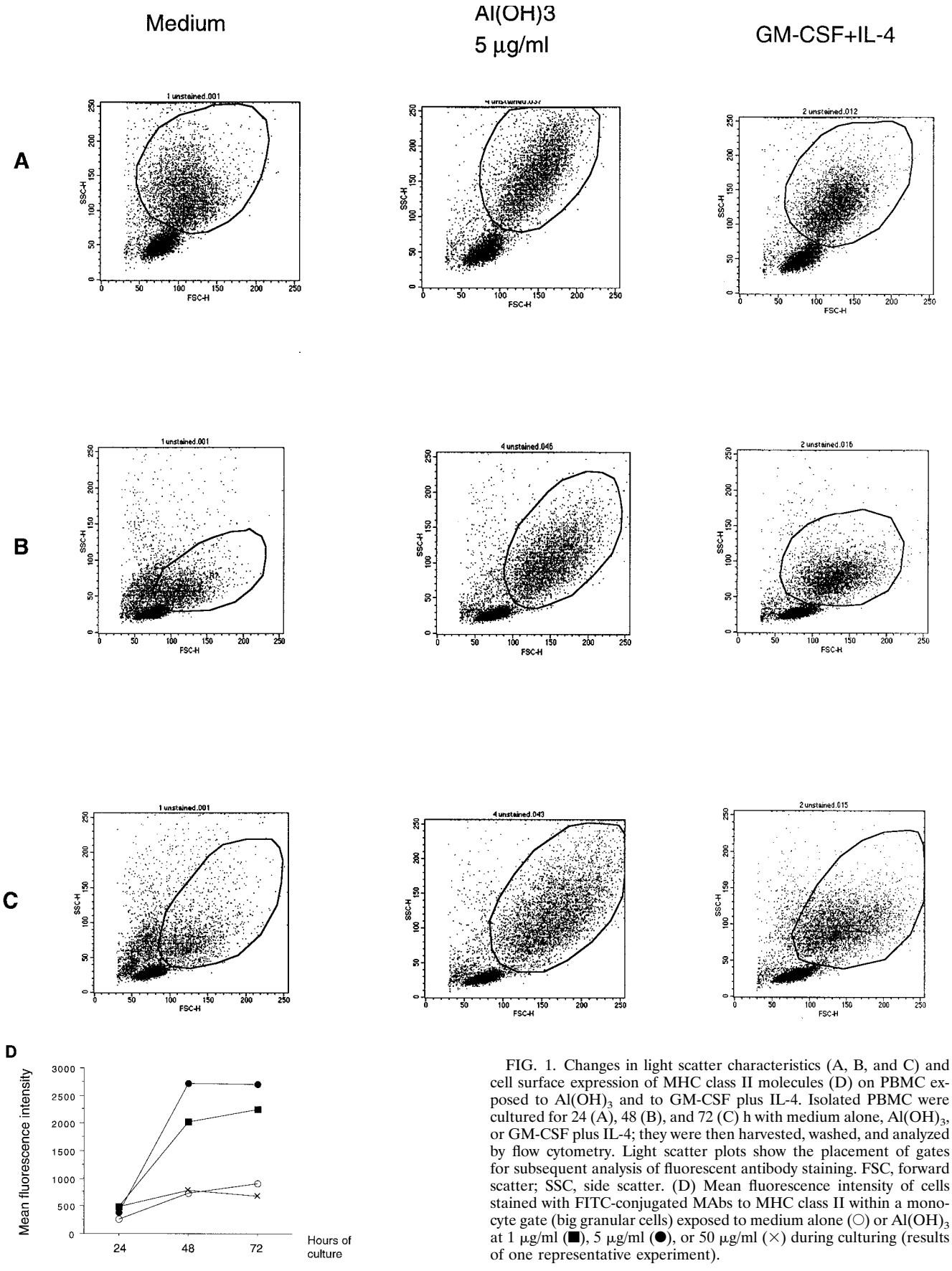

FIG. 1. Changes in light scatter characteristics (A, B, and C) and cell surface expression of MHC class II molecules (D) on PBMC exposed to $Al(OH)_3$ and to GM-CSF plus IL-4. Isolated PBMC were cultured for 24 (A), 48 (B), and 72 (C) h with medium alone, $Al(OH)_3$, or GM-CSF plus IL-4; they were then harvested, washed, and analyzed by flow cytometry. Light scatter plots show the placement of gates for subsequent analysis of fluorescent antibody staining. FSC, forward scatter; SSC, side scatter. (D) Mean fluorescence intensity of cells stained with FITC-conjugated MAbs to MHC class II within a monocyte gate (big granular cells) exposed to medium alone (○) or $Al(OH)_3$ at 1 μg/ml (■), 5 μg/ml (●), or 50 μg/ml (×) during culturing (results of one representative experiment).

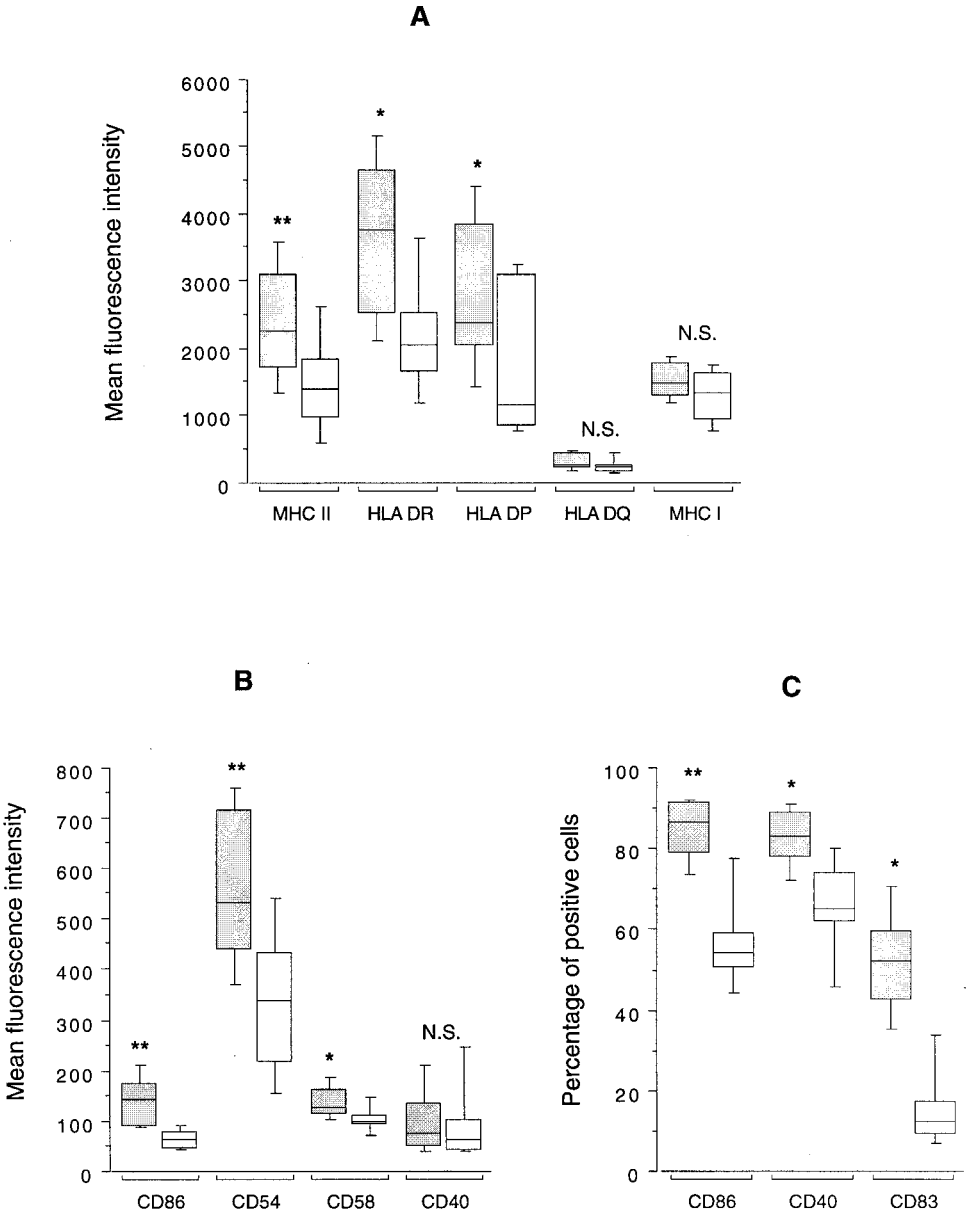

FIG. 2. Impact of $Al(OH)_3$ on the expression of MHC class II and class I and some accessory molecules. Isolated PBMC were cultured for 48 h with medium alone (empty boxes) or $Al(OH)_3$ (5 μg/ml) (filled boxes). After the culturing, the cells were harvested, washed, and analyzed by flow cytometry using FITC- or PE-conjugated MAbs. Data are mean fluorescence intensities (A and B) and the percentage of positive cells (C) within a gate of big granular cells. The results of 8 to 16 independent experiments are presented as box plots. The limits of the boxes represent the 25th and 75th percentiles of the results. The enclosed line represents the median (50th percentile), and the bars represent the 10th and 90th percentiles. The significance of differences between cultures was determined using the paired Wilcoxon signed-rank test (*, $P < 0.05$; **, $P < 0.005$).

mRNA, but not of IFN-γ or GM-CSF mRNA, was observed in cell cultures exposed to $Al(OH)_3$. To test participation of IL-4 in the up-regulation of the expression of MHC class II molecules, a neutralizing antibody specific for IL-4 was introduced into the culture of PBMC. The presence of this antibody completely abolished the aluminum hydroxide-induced increase in the expression of MHC class II expression on monocytes (Table 2), whereas the isotype-matched control antibody did not have any effect (data not shown). In contrast, neutralizing MAb specific for IFN-γ did not significantly alter the expression of MHC class II in cultures of PBMC stimulated with $Al(OH)_3$ (data not shown). These data indicated that IL-4 was specifically involved in the increase in cell surface expression of MHC class II induced by aluminum hydroxide.

Aluminum hydroxide did not influence the expression of MHC class II molecules on highly purified monocytes or monocytic cell lines. To avoid the presence of IL-4-producing cells in the culture, we used either highly purified $CD14^+$ cells (95 to 99% purity) or PBMC depleted of $CD4^+$ cells (the level of $CD4^+$ cells was less than 3% MNC) by applying a magnetic sorting technique. In both cases the effect of aluminum hydroxide on the expression of MHC class II molecules on monocytes

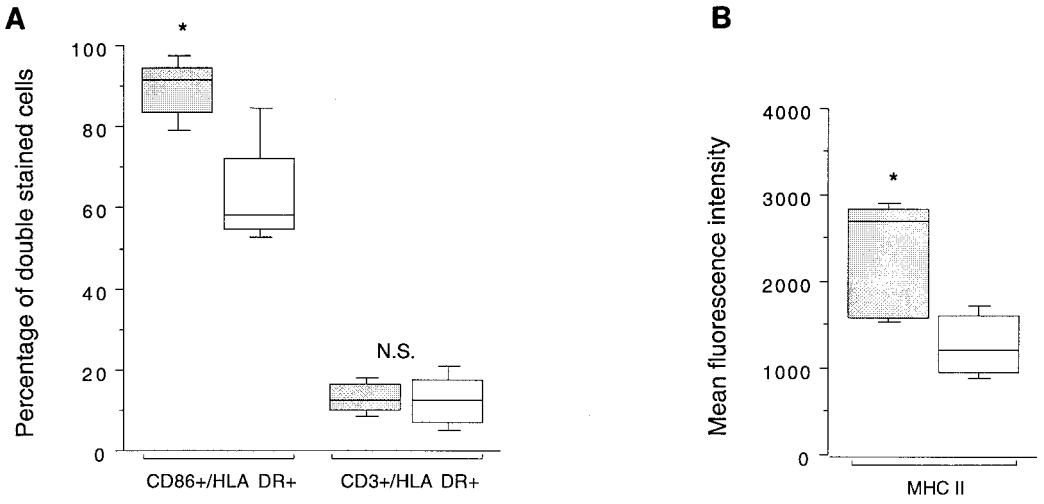

FIG. 3. Impact of $Al(OH)_3$ on the percentage of double-stained cells within the monocyte ($CD86^+$ $HLA\text{-}DR^+$) and the lymphocyte ($CD3^+$ $HLA\text{-}DR^+$) gates (A) and on MHC class II staining of $CD68^+$ cells (monocyte gate) (B). The experiment was performed as described in Fig. 2. Double immunostaining was done using FITC- and PE-conjugated MAbs. The results of 6 to 11 independent experiments are presented as box plots. Filled boxes, PBMC cultured with $Al(OH)_3$; empty boxes, PBMC cultured with medium alone. The limits of the boxes represent the 25th and 75th percentiles of the results. The enclosed line represents the median (50th percentile), and the bars represent the 10th and 90th percentiles. The significance of differences between cultures was determined using the paired Wilcoxon signed-rank test (*, $P < 0.05$).

was almost completely abolished (data not shown), indicating that T cells were the prerequisite for this phenomenon.

To further study the effects of aluminum hydroxide on pure monocyte populations, we used two monocytic cell lines representing different stages of differentiation. THP-1 is a human myelomonocytic cell line (39) which does not express MHC class II molecules unless induced to differentiate with, e.g., IFN-γ treatment (41). In contrast, Mono Mac 6 cells have phenotypic and functional characteristics of mature blood monocytes expressing MHC class II molecules and CD14 (48).

Despite their different characteristics, neither of these cell lines was found to respond to the stimulation with aluminum hydroxide with any increase in the MHC class II expression (data not shown). In contrast, stimulation with IFN-γ induced the MHC class II expression on THP-1 cells.

DISCUSSION

In the present study we made an attempt to elucidate the mechanisms underlying the potent immunoadjuvant effect of aluminum hydroxide. The function of this major vaccine adjuvant for human use is still poorly understood. The original explanation of a "depot effect" proposed in the 1930s (14) was later challenged, when it was demonstrated that excision of the complex of antigen and aluminum gel from a subcutaneous injection site shortly after injection did not diminish the immune response (19). Other studies implicated that T-cell-dependent mechanisms were involved, at least in attracting eosinophils to the injection site (35, 43). It was hypothesized that particles of aluminum, which easily bind to proteins, can be covered by immunoglobulins in situ and then may activate mast cells through Fc receptors (8). The cross-linking of Fc receptors on mast cells would then induce production of Th2-type cytokines like IL-4 and IL-5 (33). However, this idea has never been experimentally proved.

In an attempt to elucidate the properties of aluminum adjuvants, we exposed isolated PBMC from normal individuals to aluminum hydroxide. We made an attempt to make the concentration of aluminum hydroxide in cell cultures close to the vaccine content (i.e., 5 mg/ml), but it had to be greatly reduced because of its toxic effect. In our experiments, aluminum hydroxide applied at a concentration of 5 μg/ml did not affect cell viability and rapidly stimulated antigen-presenting cells. However, the actual amount of aluminum hydroxide in regional lymph nodes, where interaction between antigen-presenting cells and T cells occurs, is unknown.

After 48 h of in vitro exposure of human PBMC to this adjuvant, a large increase in the monocytic expression of surface molecules implicated in antigen presentation and T-cell activation occurred. Cultured monocytes then acquired high cell surface expression of MHC class II and B7-2 (CD86) and

TABLE 1. Aluminum hydroxide-induced expression of mRNA for monocyte-derived and Th2 cytokines[a]

Cytokine	mRNA expression (densitometry units)	
	Medium alone	Aluminum hydroxide
IL-1α	0.78 (0–4.1)	3.06 (1.7–5.8)
IL-1β	0.22 (0.21–0.23)	0.7 (0.35–1.06)
IL-2	0.07 (0–0.9)	0 (0–0.7)
IL-4	0.84 (0–1.7)	15.5 (7.1–24.0)
IL-5	0.07 (0–0.9)	0 (0–0.7)
IL-6	0 (0–0)	1.56 (0.75–2.84)
IL-10	2.9 (1.0–6.0)	4.95 (2.2–22.4)
TNF	0.2 (0.16–1.1)	1.12 (0.43–2.5)
IFN-γ	0.63 (0.44–0.82)	1.06 (0.9–1.2)
GM-CSF	0.03 (0–0.08)	0.03 (0–0.16)
TGF-β	3.0 (2.6–3.3)	2.5 (1.6–3.4)

[a] Isolated PBMC (2×10^6 cells/ml) were incubated with medium alone or $Al(OH)_3$ at 5 μg/ml. After 24 h of culturing, total mRNA was extracted and analyzed by semiquantitative RT-PCR. The densitometry units were calculated by dividing the values of the specific PCR products by the values of G3PDH PCR products. Results of four independent experiments are shown. Data are median values (minimum and maximum values are in parentheses).

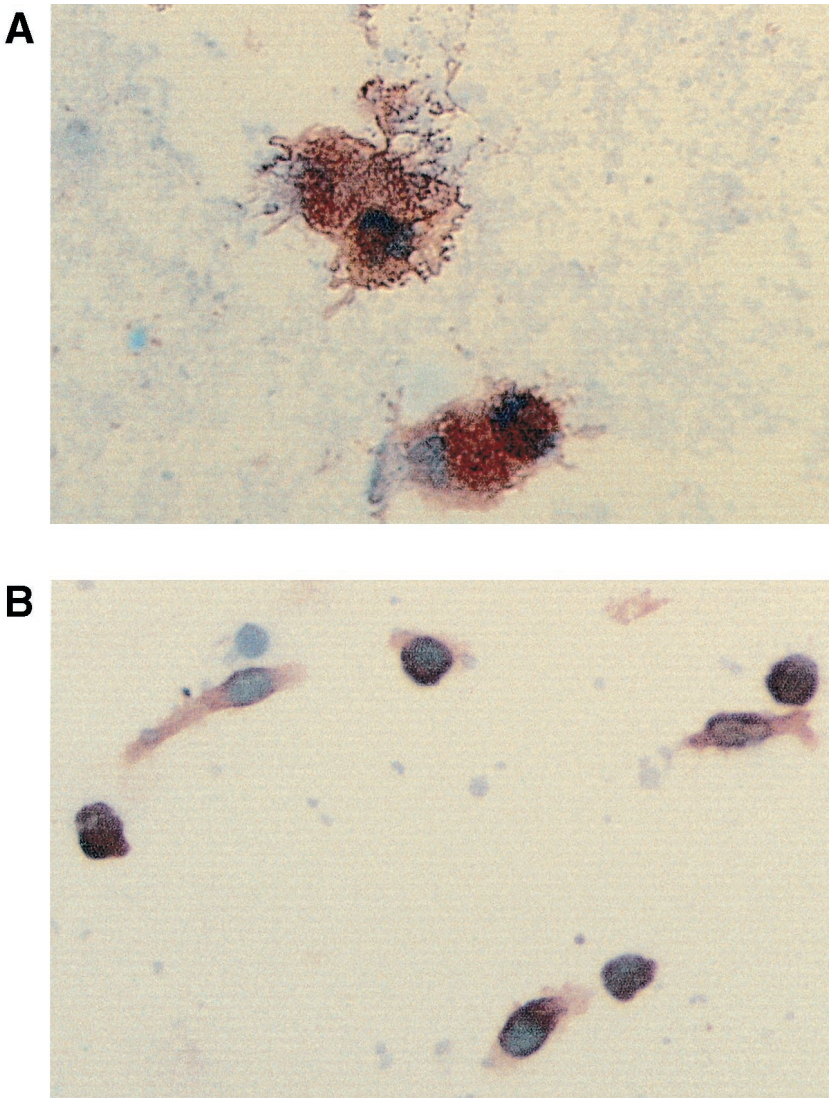

FIG. 4. Immunocytochemistry of adherent MNC cultured in plastic chamber slides for 48 h with $Al(OH)_3$ (5 μg/ml) (A) or medium alone (B). After the culturing, the slides were gently rinsed with warm PBS, allowed to dry, and fixed in ice-cold acetone. Immunostaining was performed using a MAb specific for MHC class II molecules (CR3/43) and the avidin-biotin complex peroxidase technique. Magnification, ×200.

became $CD83^+$, which is considered characteristic of mature dendritic cells (5, 18, 46, 47). Also, an increase in the expression of the costimulatory and adhesion molecules ICAM-1 (CD54), LFA-3 (CD58), and CD40, as observed in our study, has been attributed to mature dendritic cells (7). In addition, monocytes stimulated with $Al(OH)_3$ developed dendritic morphology, as revealed by immunocytochemistry analysis. The observed results were not due to an increased number of cells, since $Al(OH)_3$ did not induce proliferative responses as estimated by [^{3}H]thymidine incorporation (data not shown).

Usually the process of maturation of dendritic cells in the culture requires 5 to 7 days of stimulation of monocytes by a combination of GM-CSF and IL-4 followed by an additional 2 to 3 days of activation with either proinflammatory cytokines like IL-1, TNF, and IFN-α or lipopolysaccharide (37, 46). Such features of mature dendritic cells were acquired by cultured monocytes after 48 h in the presence of $Al(OH)_3$.

Stimulation of PBMC with aluminum hydroxide induced an increase in IL-1α, IL-1β, IL-6, and TNF mRNA expression. Above cytokines are typically produced by activated monocytes/macrophages and dendritic cells (9, 42). Moreover, IL-1α, IL-1β and TNF are known to induce the maturation of dendritic cells (37). An increased synthesis of certain Th2-type cytokine mRNAs, including those for IL-4 and IL-6, was observed, which is of potential significance in differentiation of B cells into antigen-specific antibody-secreting cells. In contrast, typical Th1-type cytokines like IL-2 and IFN-γ were not induced by stimulation with aluminum hydroxide. mRNA for GM-CSF, an important cytokine directing maturation of dendritic cells (1, 37), was virtually absent in our experiments. Hence, we observed the selectivity in the aluminum hydroxide's effect on PBMC with respect to cytokine profile.

Importantly, mRNA for IL-4 was detected in all cell samples exposed to $Al(OH)_3$. This cytokine can be produced in the cultures of PBMC by activated Th2, Th0, and NK T cells (27, 45) and is rapidly taken up by different types of cells expressing

TABLE 2. Up-regulation of MHC class II molecules induced by aluminum hydroxide is abolished in the presence of neutralizing IL-4 antibody[a]

Molecule	Mean fluorescence intensity		
	Medium	Aluminum hydroxide	Aluminum hydroxide + anti-IL-4
MHC class II	2,655.3 (627.4–2,883.0)	3,490.0 (1,848.2–3,579.5)	1,965.4 (663.8–2,734.0)
HLA-DR	2,530.4 (1,010.9–4,050.0)	3,465.3 (2,278.7–4,652.0)	2,453.5 (1,046.1–3,861.0)
HLA-DP	3,047.0 (763.5–3,248.7)	3,655.0 (2,258.0–4,420.8)[b]	2,529.3 (930.4–3,046.0)
HLA-DQ	336.3 (140.6–532.0)	372.2 (246.5–498.0)	261.3 (124.6–398.0)

[a] Isolated PBMC were cultured for 48 h with medium alone. $Al(OH)_3$, plus anti-IL-4. The experiment was performed as described for Fig. 2. The mean fluorescence intensity of cells stained with FITC- or PE-conjugated MAbs within a gate of big granular cells was determined. Data are median values (minimum and maximum values are in parentheses) of four independent experiments.

[b] Significantly different ($P < 0.05$) from the value for PBMC cultured with medium only, as calculated by the paired Wilcoxon signed-rank test.

IL-4 receptors (22, 30). Therefore, detection of this cytokine in cell culture supernatants is often problematic. IL-4 is a pleiotropic cytokine involved in regulation of different immunological functions (28), in particular via inducing MHC class II expression on monocytes (38). It was previously demonstrated that IL-4 up-regulates the cellular expression of HLA-DR and HLA-DP but not HLA-DQ molecules (13). In our experiments we observed a significant increase of cellular expression of HLA-DR and HLA-DP, but not HLA-DQ, upon exposure to aluminum hydroxide, implying that this effect could be mediated by IL-4. The simultaneous decrease in the expression of a common monocyte marker, CD14, could be also due to IL-4, since this cytokine is known to down-regulate transcription of CD14 mRNA (24).

Other cytokines known to increase cellular expression of MHC class II proteins on monocytes are IFN-γ, IL-13, and GM-CSF (6, 10, 12). IFN-γ is a potent activator of all the three HLA class II gene products and of HLA class I products as well (11, 23). However, we found no significant increase of either HLA-DQ or MHC class I expression in our system. This finding together with the lack of IFN-γ mRNA induction indicated that this cytokine was not involved in $Al(OH)_3$-triggered up-regulation of MHC class II. In addition, the presence of a MAb specific for IFN-γ in the cultures exposed to $Al(OH)_3$ did not significantly influence the expression of MHC class II. GM-CSF induces the expression of HLA-DR and -DP molecules as well as of CD1a, -b, and -c (13, 21). Since the expression of CD1 molecules was not affected by aluminum hydroxide (40), the participation of this cytokine can also be ruled out. Neither was mRNA for GM-CSF induced by aluminum hydroxide.

Our neutralization experiments provided direct evidence that IL-4 was the major factor mediating the increase in the expression of MHC class II molecules induced by aluminum hydroxide. In addition, cultures of highly purified monocytes and CD4-depleted mononuclear cells did not show increased expression of MHC class II upon exposure to $Al(OH)_3$. Experiments with the monocytic cell lines THP-1 and Mono Mac 6 further supported the results obtained from cultures of purified peripheral blood monocytes. Addition of human recombinant IL-4 to the cultures did not influence the expression of MHC class II molecules on THP-1 and Mono Mac 6 cells, although the same concentration of this cytokine induced their up-regulation on peripheral blood monocytes (data not shown). For interpretation of these results, however, it should be kept in mind that the studied monocytic cell lines constitutively have low levels of the γ chain of the IL-4 receptor complex (31, 44), which may affect the efficiency of signaling through the receptor.

Interestingly, in cultures of adherent cells the effect of aluminum hydroxide on the MHC class II expression was not diminished compared to the effect in whole PBMC, but rather more pronounced. The cultures of adherent cells, although enriched for monocytes, still contained a considerable portion of T cells (13 to 15% MNC). The remaining T cells could then produce a sufficient amount of IL-4 to induce higher expression of MHC class II molecules on monocytes in the culture.

Our results suggest that aluminum hydroxide primarily activates monocytes to produce proinflammatory cytokines and to increase the surface expression of costimulatory and adhesion molecules. Given the well-known involvement of NF-κB in the regulation of the transcription of IL-1α, IL-1β, IL-6, and TNF, as well as of B-7 and ICAM-1 genes, we suggest that NF-κB could be directly activated by aluminum hydroxide. In addition, we show that aluminum hydroxide induces production of IL-4, which in turn increases the expression of MHC class II on the antigen-presenting cells. Due to these changes, the accessory cells become functionally more efficient in antigen presentation, possibly representing the major mechanism of the in vivo immunoadjuvant effect of aluminum hydroxide.

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