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Immunology

Mature human eosinophils have the capacity to express HLA-DR

(granulocyte–macrophage colony-stimulating factor/polymorphonuclear leukocytes/class II major histocompatibility complex proteins)

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ABSTRACT **Human eosinophils are known to lose Ia antigen expression as they mature, and, accordingly, eosinophils obtained from the blood of five eosinophilic donors and three of four normal donors failed to display the major histocompatibility complex class II antigen HLA-DR, as determined by flow cytometry. However, when eosinophils from these nine donors were maintained in culture with recombinant human granulocyte–macrophage colony-stimulating factor and murine 3T3 fibroblasts, HLA-DR consistently developed on the eosinophils. By days 4–6 of culture, 24–97% of eosinophils were HLA-DR$^+$, and the eosinophils remained morphologically mature. In contrast, another class II antigen, HLA-DQ, was not detectable by flow cytometry on eosinophils from eight of nine donors. Cultured eosinophils were able to synthesize HLA-DR, as documented by the incorporation of [^{35}S]methionine into immunoprecipitable HLA-DR heavy and light chains. These findings show that mature eosinophils can synthesize and express HLA-DR and provide a means whereby eosinophils may interact with CD4$^+$ lymphocytes.**

Although eosinophils are notable participants in immunologic responses during allergic and helminthic parasitic diseases, a cooperative mechanism for eosinophil stimulation of lymphocytes has not been defined. Class II major histocompatibility complex (MHC) genes encode proteins that can be expressed on antigen-processing cells, such as macrophages, dendritic cells, and B lymphocytes, as well as on activated T lymphocytes (reviewed in refs. 1 and 2). The class II MHC protein HLA-DR mediates the MHC-restricted interactions of antigen-presenting cells with CD4$^+$ lymphocytes (1–3). Although class II proteins are present on early granulocyte precursor cells in the bone marrow, both human eosinophils (4) and neutrophils (5), with differentiation and maturation, become uniformly HLA-DR$^-$. We have found that HLA-DR$^-$ peripheral blood eosinophils synthesize and express HLA-DR while maintained in culture with recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) and murine fibroblasts. The inducibility of HLA-DR on eosinophils could provide a mechanism for eosinophils to interact with, and potentially present antigen to, CD4$^+$ lymphocytes.

MATERIALS AND METHODS

Purification of Human Eosinophils. Peripheral blood eosinophils were obtained from four normal donors (2–4% blood leukocytes were eosinophils) and four eosinophilic donors [two with *Loa loa* filariasis (17% and 52–63% eosinophils) and two with idiopathic hypereosinophilia (46% and 11% eosinophils)] and enriched to 71–94% purity by Percoll (Pharmacia) density gradient centrifugation (6). Eosinophils were also obtained by leukapheresis from another eosinophilic donor [idiopathic hypereosinophilic syndrome (50–66% eosinophils)] and enriched to 65–93% purity by sedimentation over Ficoll/Hypaque (Pharmacia).

Culture of Eosinophils. Enriched eosinophils (1.5×10^6) were cultured in 3 ml of RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and 150 units (≈50 pM) of rhGM-CSF (expressed in yeast and purified to homogeneity; Genzyme) with an adherent monolayer of Swiss 3T3 fibroblasts (American Type Culture Collection) at 37°C (5% CO_2/95% air) as described (7); 1.5 ml of culture medium was exchanged with fresh RPMI 1640/10% fetal calf serum/rhGM-CSF on days 2, 5, and 7. Nonadherent cells were collected on the indicated days; prior to flow cytometry, cell viability (>90%) was assessed with trypan blue and cell morphology was assessed by staining with phloxine–methylene blue and fast green–neutral red stains for eosinophils (8).

Flow Cytometric Analyses. Flow cytometry (FACScan; Becton Dickinson) of 10^4 cells was performed after 5×10^5 eosinophils were stained with either 10 μl of fluorescein isothiocyanate-conjugated monoclonal antibodies for HLA-DR or CD5 (anti-Leu-1), both IgG κ2a light chain (Becton Dickinson). Antigen expression was also assessed with fluorescein isothiocyanate-conjugated monoclonal antibodies (Becton Dickinson) for HLA-DQ, CD16 (anti-Leu-11a), and anti-Leu-M3. For comparison, HLA-DR expression was also evaluated on activated monocytes that had been isolated from peripheral blood, purified by adherence, and incubated for 24 hr with γ-interferon (500 units, Sigma) as described (9).

Biosynthetic Labeling of HLA-DR. Eosinophils (8.7×10^6, >99% eosinophils, 60.4% HLA-DR$^+$) from a donor with eosinophilia were harvested on day 15 of culture with rhGM-CSF and 3T3 cells. After washing with phosphate-buffered saline, eosinophils were resuspended in 1 ml of Hanks' balanced salt solution and pulse-labeled for 1 hr with 700 μCi (1 μCi = 37 kBq) of [^{35}S]methionine (Tran^{35}S-label; ICN) at 37°C (5% CO_2/95% air). Eosinophils were disrupted in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 8.0/150 mM NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% NaDodSO$_4$) for 10 min at 4°C. After centrifugation (16,000 × *g* for 15 min at 4°C), the soluble extract was immunoadsorbed in parallel with 0.6 μg of control monoclonal antibody (UPC 10; Organon Teknika-Cappel) or 5 μl of anti-HLA-DR-specific (LB3.1) monoclonal antibody (10). After 2 hr at 4°C, 50 μl of protein A-Sepharose (Genzyme) was added for 45 min. Protein A-Sepharose complexes were washed three times with RIPA buffer and once with water and then were boiled for 3 min in Laemmli sample buffer (11). Proteins were resolved by NaDodSO$_4$/PAGE (1% NaDodSO$_4$, 10% polyacrylamide) and visualized

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Abbreviations: MHC, major histocompatibility complex; rhGM-CSF, recombinant human granulocyte–macrophage colony-stimulating factor.

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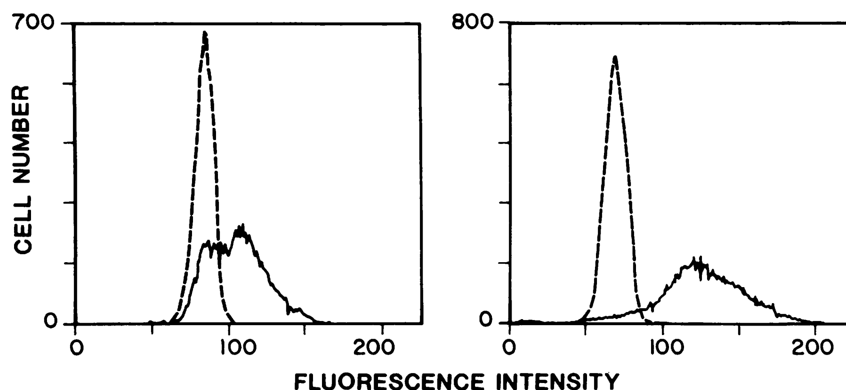

FIG. 1. Flow cytometry of eosinophils obtained after 5 days of culture with rhGM-CSF and 3T3 fibroblasts and labeled with control anti-CD5 (---) or anti-HLA-DR (—) monoclonal antibodies. (*Left*) Cells isolated from a donor without blood eosinophilia. (*Right*) Cells obtained from a donor with the idiopathic hypereosinophilic syndrome. Linear fluorescence intensity is in arbitrary units.

by fluorography of gels exposed for 14 days at room temperature with Kodak X-Omat film.

RESULTS

Eosinophils, obtained from nine donors (five with and four without blood eosinophilia) and maintained in culture with adherent murine fibroblasts and rhGM-CSF, were shown by flow cytometry to express HLA-DR (Fig. 1). Peripheral blood eosinophils purified from eight of these nine donors were HLA-DR$^-$ prior to culture. As evaluated in 14 studies, after 4–6 days in culture, eosinophils from each donor became HLA-DR$^+$ (24–97%) (Table 1). Eosinophil cell-surface expression of HLA-DR was evident by day 2 of culture and was maximal by days 3–7 (Fig. 2). In contrast, another class II MHC antigen, HLA-DQ—also not detectable on eosinophils before culture, was expressed on cultured eosinophils from only one donor (Table 1).

As assessed on day 5 of culture, the average mean channel fluorescence above background for the HLA-DR$^+$ eosinophils was 45.9 (range, 28.6–65.0). For the eight cultures in which >75% of eosinophils were HLA-DR$^+$, eosinophil staining showed a unimodal distribution (e.g., Fig. 1 *Right*) with an average mean channel fluorescence above background of 48.7, with HLA-DR staining extending for an average of 49.3 fluorescence units above the mean. In the other six cultures, the mean fluorescence intensity was 42.2 for HLA-DR$^+$ eosinophils, with HLA-DR staining extending an average 61.7 fluorescence units above the average mean intensity. In these cultures, there was a bivariate distribution of HLA-DR$^+$ and HLA-DR$^-$ eosinophils (e.g., Fig. 1 *Left*). In comparison, γ interferon-activated monocytes showed a unimodal distribution of HLA-DR expression with the mean channel fluorescence above the background of 103, with HLA-DR staining extending ± 55 fluorescence units above and below the mean.

The identity of the cultured HLA-DR$^+$ cells as eosinophils was ascertained by light microscopy and flow cytometry. By day 5 of culture, >95% of the nonadherent cells were eosinophils. These cells had the morphologic and tinctorial features of eosinophils by Wright–Giemsa, neutral red–fast green (8) and phloxine–methylene blue (8) staining. Specific staining of eosinophil cytoplasmic granules was demonstrable with fast green and phloxine stains. Furthermore, the cultured eosinophils were morphologically mature, with condensed, segmented, primarily bi-lobed nuclei as reported (7). Contaminating neutrophils, representing 6–35% of leukocytes at the start of cultures (Table 1), do not survive under these culture conditions (7) and were not detectable by days 4–6 by cytologic staining or by flow cytometry with anti-CD16 monoclonal antibody. In addition, the absence of other cells known to express HLA-DR was corroborated by stain-

Table 1. Blood eosinophilia of donors and HLA-DR and other antigen expression on cultured eosinophils

	Eosinophils			Antigen expression on day(s) of culture, %						
	Donor blood		Pre-culture enrichment,	HLA-DR$^+$		HLA-DQ$^+$		CD5$^+$		Leu-M3$^+$
Donor	no./μl	%	% on day 0	0	4–6	0	4–6	0	4–6	0–2
1 a	11,150	66	93	2.4	28.6	—	2.0	1.7	1.5	2.4
b	12,260	63	90	0.8	24.5	0.7	0.8	0.4	1.6	—
c	2,020	50	65	1.0	42.2	0.7	2.6	0.5	3.5	1.1
2 a	7,300	52	71	0.9	23.9	1.0	4.7	0.8	3.5	0.6
b	10,300	63	94	2.8	33.9	2.7	4.5	1.3	2.4	1.7
3 a	≈13,800	46	93	3.4	94.4	3.4	9.8	0.7	1.4	0.8
4 a	≈1,870	11	67	0.7	41.3	1.1	0.8	0.4	0.6	0.7
5 a	960	17	73	4.4	91.6	—	1.7	2.3	1.6	2.8
6 a	≈400	4	79	1.1	75.5	0.4	1.7	0.4	1.9	0.3
b	≈500	5	90	7.5	97.3	4.0	—	3.2	1.5	2.3
7 a	≈200	2	80	1.5	54.6	1.2	2.9	1.5	2.6	4.3
b	≈300	3	72	—	50.5	—	—	—	1.0	—
8 a	≈200	2	71	23.6	97.4	—	4.6	1.0	2.7	0.8
9 a	≈500	5	67	1.8	93.5	—	4.7	0.5	1.1	3.1

Eosinophils were obtained from five eosinophilic donors and four normal donors with blood eosinophil counts and percentages as noted. Eosinophils were enriched to the purities indicated prior to culture (day 0) and then cultured with rhGM-CSF. Antigen expression was measured by flow cytometry with fluoresceinated monoclonal antibodies for HLA-DR, HLA-DQ, CD5 (T cells), and anti-Leu-M3 (monocytes/macrophages). Background fluorescent staining, defined by staining with anti-CD5, has not been subtracted.

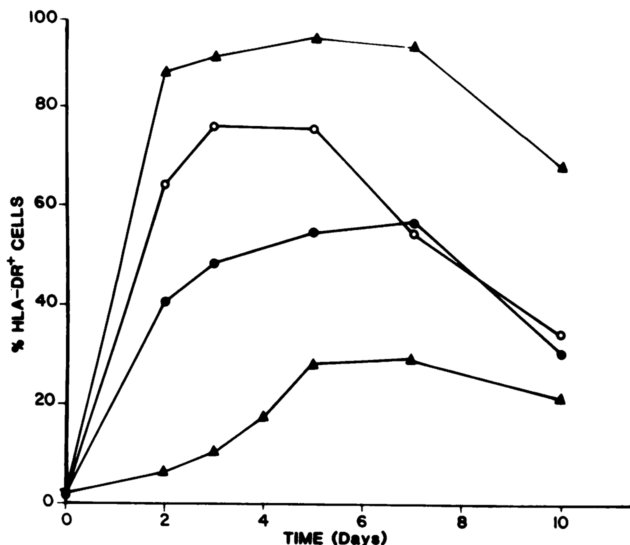

FIG. 2. Time course of expression of HLA-DR by eosinophils during culture. Eosinophils from two donors with (▲, △) and two without (●, ○) blood eosinophilia were purified, cultured with rhGM-CSF and 3T3 fibroblasts, and examined by flow cytometry for HLA-DR expression. Percentage of HLA-DR$^+$ cells includes background staining, which was ≤3.5% as assessed with anti-CD5 antibody.

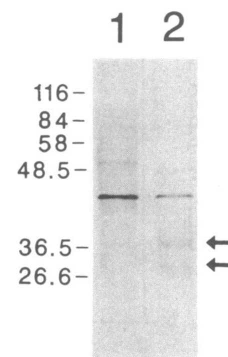

FIG. 3. NaDodSO$_4$/PAGE of HLA-DR heavy and light chains (arrows) immunoprecipitated from cultured [^{35}S]methionine-labeled eosinophils with either nonspecific control (lane 1) or specific anti-HLA-DR (lane 2) monoclonal antibodies. Molecular mass standards are shown in kDa.

ing cultured cells with monoclonal antibodies for monocytes and T cells (Table 1).

The specificity of HLA-DR expression on cultured eosinophils was confirmed with a second HLA-DR-specific monoclonal antibody, LB3.1, which immunoprecipitates both chains of HLA-DR (10). From extracts of cultured eosinophils labeled with [^{35}S]methionine in the absence of fibroblasts, this monoclonal antibody precipitated proteins with molecular masses of 27.5 and 32.4 kDa (Fig. 3), consistent with the light and heavy chains of HLA-DR (10). The same findings were obtained with eosinophils from donors with and without eosinophilia.

DISCUSSION

Ia antigen (4) and specifically HLA-DR (12) are expressed on eosinophil colony-forming cells. Since class II MHC antigens are absent from more mature eosinophils, the expression of HLA-DR on granulocytes has been taken as a differentiation marker, with expression limited to the earliest stages of development (4). Thus, the effect of culturing mature human HLA-DR$^-$ eosinophils with rhGM-CSF and fibroblasts was notable for the *de novo* expression of an MHC class II antigen on a type of leukocyte not previously recognized to express such antigens. HLA-DR was absent on mature eosinophils circulating in the blood of all but one of the normal and eosinophilic donors but was expressed by these mature eosinophils maintained in culture with rhGM-CSF. Further, the incorporation of [^{35}S]methionine into immunoprecipitable HLA-DR heavy and light chains indicated that there was new synthesis of HLA-DR by eosinophils and not simply its mobilization from an intracellular pool for cell-surface expression.

GM-CSF, which can be produced by T lymphocytes, monocytes, fibroblasts, and endothelial cells (13–15), has been recognized to stimulate functions of mature eosinophils (16), including antibody-dependent cytotoxicity (17, 18), synthesis of leukotriene C_4 in response to calcium ionophore stimulation (17), and cell-surface expression of antigens granulocyte function antigen 1 (GFA-1) and Mo1 (18). High-affinity receptors ($K_d \approx 2.3 \times 10^{-11}$) for GM-CSF have been demonstrated on eosinophils (19). Further, GM-CSF prolonged eosinophil survival in culture (18), and its presence was required to maintain the viability of eosinophils cultured with 3T3 fibroblasts (7). Utilizing the coculture system with 3T3 fibroblasts and GM-CSF, we have shown that eosinophils during *in vitro* culture can express HLA-DR. Because both fibroblasts and GM-CSF were needed to maintain eosinophil viability in culture as noted before (7), the specific roles of GM-CSF and fibroblasts in eliciting HLA-DR expression by eosinophils could not be determined. The 50 pM concentration of rhGM-CSF used would be in accordance with the dissociation constant of eosinophil GM-CSF receptors and within the range of GM-CSF concentrations eliciting physiological responses (19). On other cell types, GM-CSF has augmented MHC class II Ia antigen expression on adherent murine spleen cells (20), enhanced the accessory cell function of murine spleen and epidermal Langerhans cells (20, 21), and increased the Ia expression and Ia-related functions of murine macrophages (22).

Cultured eosinophils consistently expressed HLA-DR but not HLA-DQ. A similar predominance of HLA-DR expression has been noted on other cell types including monocytes (23), Kupffer cells (24), and synovial lining cells (25). Differential regulation of expression of these two MHC class II proteins in HL-60 myelomonocytic cells has been noted (26), but the mechanism and significance of the predominant HLA-DR expression by eosinophils, as well as by other cell types, are not defined. The mean fluorescence intensity of HLA-DR expression on eosinophils was less than that on γ interferon-stimulated monocytes, but for eosinophils from all cultures, there was considerable overlap in fluorescent HLA-DR staining between the HLA-DR$^+$ eosinophils and the activated monocytes. Other cytokines, alone or in concert, may elicit greater expression of HLA-DR on eosinophils.

The circulating blood eosinophils from eosinophilic donors included eosinophils of normal and diminished (hypodense) density. Donor 1, for instance, on two separate occasions had >95% hypodense eosinophils as assessed with metrizamide density gradients (7) (data not shown). The absence of HLA-DR on the cells from eosinophilic donors would suggest that HLA-DR expression is not an early marker for the apparently activated hypodense eosinophils (7, 27), although HLA-DR expression might develop after these cells leave the circulation for more prolonged residence in tissues (28). Quantitatively >99% of eosinophils are present in tissues (28) where eosinophil function is subject to stimulatory cytokines (16). In response to parasitic infections, eosinophils can serve as helminthotoxic effector cells (29), and in allergic diseases eosinophils are a source of immunopathogenic mediators (27). Our findings indicate that mature, end-stage eosinophils possess the capacity for the *de novo* synthesis and expression

of HLA-DR. The expression of this class II MHC protein might enable eosinophils to have additional immunologic functions mediated through a capacity to interact with $CD4^+$ lymphocytes, and these potentially MHC-restricted activities might include functioning as antigen-processing cells.

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