Identification of Antigen-Capturing Cells as Basophils

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Binding of intact Ag is a hallmark of Ag-specific B cells. Apart from B cells, a small number of non-B cells can bind Ag with comparable efficacy as B cells and are found in the peripheral blood, spleen, and bone marrow of mice. This population has been observed for a long time and recently named “Ag-capturing cells.” Their identity remained enigmatic. In this study, we show that these cells are basophilic granulocytes. Their ability to capture Ags is dependent on surface IgE receptors and on Ag-specific plasma IgE molecules appearing after immunization. Several surface markers including surface bound IgE, IL-3R, CD45, CD16/32, and the chemokine receptor CCR2 were used to clearly identify these cells. Cross-linkage of surface Igs results in the release of large amounts of IL-4 and IL-6. The data identify basophils as Ag-capturing cells and support the concept of basophils as important regulators of humoral immune responses. The Journal of Immunology, 2005, 174: 735–741.

Materials and Methods

Immunizations

C57BL/6 mice were injected i.p. with 80 μg soluble allopehycocyanin (Prozyme) in 200 μl of PBS with 10⁷ heat-killed Bordetella pertussis. Three weeks after the first immunization, a boost dose of 80 μg soluble allopehycocyanin with B. pertussis was given. Mice were analyzed between 10 and 30 days after the boost. Governmental approval was given for the animal experiments.

Cell preparations

Blood was drawn from the retro-orbital plexus and anticoagulated with 1 mM EDTA. Plasma was obtained by centrifugation of blood for 10 min at 1200 g.
Flow cytometry

To detect allophycocyanin-binding cells, cell preparations were washed three times in ice-cold PBS and stained with 10 μg/ml allophycocyanin (Prozyme) dissolved in PBS. The following Abs were purchased from BD Pharmingen: CD45/LCA-FITC (30-F11), CD11b-FITC (M1/70), CD19-FITC (1D3), CD11c-PE (HL3), CD16/32-PE (2.4G2), CD19-PE (1D3), HLA-IA/IE-PE (M5/1L4,15,2), CD4-Cy5 (RM4–5), CD8-Cy5 (53–6.7), Gr-1-APC (RB6–8C5), c-kit-PE (2B8) CD123-PE, and Gr1-allophycocyanin. Basophils were identified by gating on the lymphocyte population, by medium expression of CD45, by high expression of surface IgG, and by absence of Gr-1. The purity of the basophils was routinely >98% by subsequent FACS analysis. For isolation of basophils from the spleen, CD19-negative cells is detectable in immunized mice. These allophycocyanin-positive CD19-negative B cells, a clear population of allophycocyanin-positive non-B cells are located within the lymphocyte region.

Isolation of basophils from peripheral blood and spleen

Basophils were prepared from unimmunized mice. Erythrocytes were lysed by addition of 0.2% sodium chloride for 10 s with subsequent correction of tonicity with an identical volume of 1.6% sodium chloride. To isolate basophils from the peripheral blood, the cells were stained with CD45-FITC, CD16/32-PE, and Gr1-APC for 30 min on ice. Basophils were identified by gating on the lymphocyte gate, by medium expression of CD45, by high expression of CD16/32, and by absence of Gr1 and were sorted on a cell sorter (MoFlo; DakoCytomation). The purity of the basophils was routinely >98%. Sixty microliters of streptavidin-Sepharose (Amersham Biosciences) was added with 5% mouse serum and staining with CD45-FITC, CD16/32-PE, and Gr1-APC for 30 min on ice. Basophils were identified by gating on the lymphocyte gate, by medium expression of CD45, by high expression of CD16/32, and by absence of Gr1 and were sorted on a cell sorter (MoFlo; DakoCytomation). The purity of the basophils was routinely >98%. Eighty microliters, ~150,000 basophils were recovered from the peripheral blood and 350,000 from the spleens. Staining with anti-IgE, CD123 (IL-3R), CCR2, HLA IA/IE, CD19, B220, CD35, CD21, and CD138 Abs showed that the isolated cells were basophils.

Depletion of IgE from murine plasma

Sixty microliters of streptavidin-Sepharose (Amersham Biosciences) was loaded with 60 μg biotinylated anti-IgE Abs (clone R35-118) or biotinylated anti-IgG2a (clone R19-15) as control and washed five times to remove unbound Abs. Plasma from allophycocyanin-immunized mice (200 μl) was incubated twice for 1 h at room temperature with 30 μl of anti-IgE- or anti-IgG2a-coated Sepharose. The Sepharose was removed by centrifugation and complete depletion of IgE was shown by ELISA as described below.

Cell culture

Basophils (40,000/well) were culture in round-bottom plates for 3 days in RPMI 1640 with 10% FCS and 1% penicillin/streptomycin. Where indicated, cells were stimulated with a polyclonal rabbit anti-mouse IgG (Dianova, Hamburg, Germany).

ELISA

IL-4 and IL-6 were measured in the cell culture supernatant with a commercially available kit (OptEIA; BD Pharmingen).

For measurement of total IgE in plasma, ELISA plates were coated with anti-IgE mAb (clone R35-72; 10 μg/ml) and bound IgE was detected with a biotinylated anti-IgE mAb (clone R35-118) followed by streptavidin-HRP (DakoCytomation) according to the manufacturer’s recommendations.

To test cross-reactivity of isotype-specific Abs against murine IgG1, IgG2a, and IgG2b with purified murine IgE (BD Pharmingen), IgE was coated overnight at a concentration of 10 μg/ml followed by incubation with the biotinylated isotype-specific mAbs and streptavidin-HRP. No cross-reactivity of IgG isotype-specific Abs was detectable, while an Ab against IgE showed good reactivity with plate-bound IgE (data not shown).

Results

Detection and characterization of Ag-capturing cells

C57BL/6 mice were immunized twice with allophycocyanin using killed B. pertussis as adjuvant. Bone marrow, peripheral blood leukocytes, and splenocytes were stained with allophycocyanin and Abs against CD19 or B220. As described by Bell and Gray (3), two populations of allophycocyanin-binding cells were detected in allophycocyanin-immunized mice. In addition to allophycocyanin-positive B cells, a second population of CD19-negative/B220-negative allophycocyanin-binding cells was detectable at a frequency of 0.58% of total leukocytes in the peripheral blood, 0.77% in the bone marrow (Fig. 1a), and 0.21% in the spleen (data not shown).

FIGURE 1. Detection and characterization of allophycocyanin-binding non-B cells. a. Peripheral blood cells (left column) and bone marrow cells (right column) from C57BL/6 mice immunized twice with allophycocyanin (upper row) or control mice (lower row) were stained with CD19-PE and allophycocyanin. Apart from the small fraction of allophycocyanin-positive CD19-positive B cells, a clear population of allophycocyanin-positive CD19-negative cells is detectable in immunized mice. These allophycocyanin-positive non-B cells are more frequent in the bone marrow than in the peripheral blood. b. Light scatter properties of total peripheral blood cells (left panel) and allophycocyanin-positive non-B cells (right panel). Allophycocyanin-positive non-B cells are located within the lymphocyte region. c. The population of allophycocyanin-positive non-B cells was isolated by magnetic beads and FACS sort and stained in vitro by cross-linkage of surface IgGs for 3 days. Cytokines were measured in the culture supernatant by ELISA. Cross-linkage of surface IgGs resulted in a strong release of IL-4 and IL-6.
In the bone marrow, allophycocyanin-positive B cells were detected at a very low frequency, while allophycocyanin-positive non-B cells were much more abundant. The allophycocyanin-positive B cells show a wide range of allophycocyanin staining intensity, as expected for an oligoclonal B cell population with variable affinity and avidity for the Ag. In contrast, the allophycocyanin staining on the non-B cell compartment is very homogeneous and of identical signal strength in bone marrow, spleen, and peripheral blood. By backgating we could localize the allophycocyanin-positive non-B cells within the lymphocyte gate with somewhat higher side scatter properties than T or B cells (Fig. 1b). Costaining with several surface markers revealed that the allophycocyanin-positive non-B cells express low amounts of CD11b, but are negative for the additional B cell or plasma cell markers HLA-II, IgM, CD138, and CD21/35 (data not shown). Staining with a variety of other surface markers for T cells, NK cells, and monocytes did not result in identification of this cell population. However, a combination of staining for CD45, Gr-1, CD16/32, and surface Ig could unambiguously identify this cell population. They show reduced expression of the common leukocyte Ag CD45, comparable to neutrophils, but are negative for the neutrophil marker Gr-1. In addition, they are highly positive for CD16/32 and express large amounts of surface Iggs, as detected with a polyclonal Ab against IgG (Fig. 2b and data not shown). Combination of these surface markers enabled us to isolate the cell population in sufficient quantities for in vitro analysis from the peripheral blood and the spleen of mice with a purity of >98%. The most striking finding was the release of large amounts of IL-4 and also some IL-6 after stimulation by cross-linkage of surface Ig with a polyclonal anti-mouse IgG Ab (Fig. 1c). LPS (50 μg/ml) was used as additional stimulus but did not result in a significant release of IL-4. These functional properties were very much reminiscent of the previously described IL-4-secreting non-B non-T cells (19) and raised the possibility that the cell population might consist of basophilic granulocytes that are known to release IL-4 after stimulation (20, 21). Indeed, staining with Abs against IgE and the IL-3R (CD123) showed that all allophycocyanin-positive non-B cells were highly positive for both molecules (Fig. 3a) and that all IgE highly positive cells strongly bind allophycocyanin (Fig. 3b). Strong binding of IgE and homogeneous expression of the IL-3R is a hallmark for basophilic granulocytes that typically localize within the lymphocyte gate by light scatter properties (8, 9). It also became evident that allophycocyanin-positive cells were either CD19-positive B cells or IgE/CD123-positive basophils. The additional IgE-negative population of Ag-binding cells described by McHeyzer-Williams et al. (2) was not detectable in our mice.

We can exclude that eosinophils or mast cells contribute to the allophycocyanin-positive non-B cells. Eosinophils express only marginal levels of IL-3R (100-fold less than basophils) and are not located in the lymphocyte gate, but in the neutrophil gate (22). In addition, human eosinophils do not express the chemokine receptor CCR2 (23), while basophils do and respond to CCR2 ligands (7, 8). Staining of murine basophils for CCR2 and CCR5 reveals a high expression of CCR2 (Fig. 3c), while CCR5 was not detectable (data not shown). Expression of CCR2 was confirmed by down-modulation of the CCR2 receptor after incubation of the cells with murine macrophage chemotactic protein 1 (30 min at 37°C. Mast cells are not found in the peripheral blood and can therefore be excluded as Ag-capturing cells at least in the blood (6). In addition, we stained the cells with an Ab against c-kit that
showed a weak expression on Ag-capturing cells in all three compartments, the peripheral blood, spleen, and bone marrow. c-kit-positive cells in the bone marrow with 40-fold higher expression of c-kit than basophils did not capture allophycocyanin, indicating that the Ag-capturing cells are not mast cells. Dendritic cells could also be excluded by the absence of CD11c and HLA II on Ag-capturing cells. A summary of the analyzed surface markers is given in Table I. We also performed cytosin on the isolated basophils and stained the cells with May-Grünwald-Giemsa. The cells are slightly granular with basophilic granules and have indented nuclei with lobulation seen in some cases (data not shown). This is consistent with the morphological description of murine basophils that characteristically have few granules and a nucleus with heavily condensed chromatin in two lobes (5).

Ag capture is dependent on IgE receptors on basophils and Ag-specific IgE

We analyzed the binding of various Ig subtypes on basophils in comparison to other cell types (Fig. 2a). Using Abs against murine IgG1, IgG2a, IgG2b, and IgE, we found that basophils in the peripheral blood of BALB/c mice have several-fold more Igs bound on their surface than monocytes, neutrophils, and even B cells (Fig. 2a). These differences were most pronounced for IgE that was by far the most predominant Ig on basophils and was hardly detectable on other cell types. However, also various IgG subtypes were detectable at higher levels on basophils than on other cell types. This binding of IgG subtypes might result from a higher expression of Ig FcRs on basophils. This was indeed the case for the low- and medium-affinity FcRs for IgGs, FcyRIII, and FcyRII (CD16/32) (Fig. 2b) that would at least mediate the binding of IgG containing immune complexes. Using recently described mAbs against CD64 (18), we were not able to demonstrate CD64 (FcγRI) expression on basophils in the peripheral blood and found only a marginal expression of CD64 on basophils in the bone marrow (data not shown). However, we could demonstrate that the binding of allophycocyanin-specific Igs on basophils is very stable. When basophils from allophycocyanin-immunized mice were washed several times and incubated for 48 h in medium, no significant reduction in their ability to bind allophycocyanin was observed (data not shown). These data suggest that the capture of Igs by basophils occurs with rather high affinity or avidity.

The concept of Ab capturing by basophils was tested in vitro. Bone marrow cells from unimmunized C57BL/6 mice were incubated for 1 h at 37°C with plasma from control and allophycocyanin-immunized mice and washed three times before allophycocyanin was added. Incubation with plasma (75% of total volume) from allophycocyanin-immunized mice enabled the basophils to bind large amounts of allophycocyanin (Fig. 4, upper panel), whereas other cell types showed little allophycocyanin binding (Fig. 4, lower panel). Incubation with plasma from control mice did not allow allophycocyanin binding to basophils.

We then wanted to analyze which Ig FcRs mediate the binding of allophycocyanin-specific Igs and which Ig isotypes are responsible for the allophycocyanin-binding capacity of basophils. We therefore preincubated bone marrow cells from naive C57BL/6 mice for 1 h at 37°C with a high concentration of an IgE isotype control Ab (50 μg/ml) or a combination of IgG1, IgG2a, IgG2b, and IgG3 isotype control Abs (50 μg/ml each) to block unoccupied high-affinity IgE or IgG receptors, respectively. Without washing, cells were further incubated for 1 h at 37°C with plasma (75% of total volume) from allophycocyanin-immunized mice or naive control mice. Preincubation with IgE Abs reduced the ability of basophils to capture allophycocyanin-specific Igs by >90% (Fig. 5, a and b), whereas preincubation with the combination of IgG molecules had no effect (Fig. 5c). Preincubation with lower concentrations of IgE control Abs (5 μg/ml) also markedly reduced the ability of basophils to capture allophycocyanin-specific Igs (80% inhibition). To determine whether the isotype of the allophycocyanin-specific Igs that bind to basophils is indeed IgE, we completely depleted the IgE molecules from the plasma of allophycocyanin-immunized mice with anti-IgE-coated Sepharose. IgE-depleted plasma lost again >90% of its ability to mediate allophycocyanin binding to basophils in comparison to undepleted plasma (Fig. 6). Sepharose coated with a control Ab did not reduce the ability of plasma to mediate allophycocyanin binding (data not shown). These experiments show that IgE binding sites are required for the capture of allophycocyanin-specific Igs from mouse plasma and that allophycocyanin-specific IgE molecules are responsible for the ability of basophils to capture allophycocyanin.

The observation that basophils are able to capture allophycocyanin-specific IgE molecules during a 1-h exposure with plasma from allophycocyanin-immunized mice suggests that in unimmunized mice the IgE binding sites on basophils are not completely occupied and/or that the exposure of basophils with IgE induces an up-regulation of FcεRI and prevents the removal of unoccupied FcεRI from the cell surface (24–26). As expected, the amount of IgE molecules bound to basophils from unimmunized mice increased after exposure to IgE for 1 h at 37°C. The mean channel

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**FIGURE 4.** Ag capture by basophils in vitro. Bone marrow cells from unimmunized control mice were incubated for 1 h at 37°C with plasma (75% of volume) from unimmunized control mice (gray area) or allophycocyanin-immunized mice (white area) and allophycocyanin binding was measured by flow cytometry. Basophils (upper panel) were identified by high expression of IgE and absence of CD19. All cells except basophils are shown in the lower panel. After incubation with plasma from allophycocyanin-immunized mice, only basophils show a strong binding of allophycocyanin, while incubation with control plasma does not allow allophycocyanin binding.
fluorescence for IgE bound to these basophils was 447 after exposure to IgE-depleted plasma, 824 after exposure with an IgE isotype control Ab (50 μg/ml; IgE pre) or PBS for 1 h and without washing further incubated for 1 h at 37°C with plasma (75% of total volume) from naive control mice (Co-pl.) or allophycocyanin-immunized mice (APC-pl.). Allophycocyanin binding on basophils identified by high expression of IgE and absence of CD19 was quantified by flow cytometry. Depletion of basophils with IgE reduced the capture of allophycocyanin-specific IgE by >90%. Preincubation of bone marrow cells with a combination of murine IgG Abs (IgG1, IgG2a, IgG2b; 50 μg/ml each) as described above did not influence the ability of basophils to capture allophycocyanin-specific IgE.

Since IgE induced up-regulation of surface FcERI is supposed to occur only at 37°C, we also analyzed whether the capture of allophycocyanin-specific IgE molecules from the plasma of immunized mice is higher at 37°C than on ice. Incubation of basophils from unimmunized mice with plasma from allophycocyanin-immunized mice and after incubation with plasma from unimmunized control mice. The increased binding of IgE by basophils after incubation with plasma from unimmunized mice compared with control mice is consistent with ELISA data measuring the total amount of IgE in the plasma. Immunized mice have ~8-fold higher IgE levels in their plasma than unimmunized mice (4.6 vs. 0.55 μg/ml). It is reasonable to assume that a fraction of these additional IgE molecules will have specificity for allophycocyanin and mediate the ability of basophils to capture Ag.

Discussion

After immunization of mice with Ags (e.g., fluorescent proteins like allophycocyanin) a very small fraction of leukocytes is able to bind intact Ags on the cell surface. Although low binding of intact Ag is detectable on a larger fraction of leukocytes (e.g., monocytes and neutrophils), strong binding is only observed on two distinct cell populations. One cell population consists of the small subpopulation of Ag-specific CD19-positive B cells. Ag binding on these B cells is inhomogeneous and somewhat higher than on the second cell population that was described in several manuscripts without being identified. In a recent report, Bell and Gray (3) demonstrated that these cells passively capture IgE on their surface and cannot be considered as a new population of CD19 and B220-negative memory B cells as previously suggested (2). We were now able to demonstrate that the Ag-capturing cells are basophilic granulocytes that capture Ag-specific IgE on their cell surface and thereby bind the Ag. We further show that the binding of Ag is dependent on surface IgE receptors and Ag-specific IgE in the plasma of immunized mice.

Several parameters were used to identify these cells as basophils. By light scatter properties in flow cytometry they are located in the lymphocyte gate with a somewhat higher side scatter than the majority of B or T cells. The Ag-capturing cells are negative for B cells markers (CD19, B220, CD21/35, IgM, HLA II) and the plasma cell marker CD138 (Syndecan-1). They show a high expression of surface IgE and the IL-3R CD123, and release large quantities of IL-4 after cross-linkage of surface IgE. In the peripheral blood, bone marrow, and spleen, only one cell population with high expression of IgE and CD123 was detectable. This population was identical to the allophycocyanin binding non-B cells, which means that all basophils identified by a high surface expression of IgE and CD123 were allophycocyanin-positive and that all allophycocyanin-positive non-B cells homogeneously express high levels of surface IgE and CD123. We also performed co costing with CD11c and HLA-II to exclude dendritic cells as Ag-capturing cells, as some dendritic cell subpopulations have been described to express the IL-3R (27–29). In addition, we excluded mast cells as Ag-binding cells. Allophycocyanin-positive
cells in all three compartments (blood, spleen, bone marrow) expressed only marginal levels of c-kit (mean channel fluorescence, \(\sim 25\)) and cells in the bone marrow that were clearly c-kit-positive (mean channel fluorescence, \(\sim 1000\)) did not capture allophycocyanin. Further evidence that the allophycocyanin-binding non-B cells are basophilic granulocytes comes from light microscopy that showed slightly granular cells with basophilic granules and indented nuclei with lobulation. We also analyzed the expression of the chemokine receptors and found a high expression of CCR2 and an absence of CCR5, a feature that has also been described for human basophils.

We then wanted to know why only basophils and not other FcR-positive leukocyte subsets (like monocytes or neutrophils) are able to capture Ag-specific Igs and bind intact Ag. We therefore analyzed the amount of surface-bound Igs on basophils compared with other cell types. Using isotype-specific Abs against IgM, IgG1, IgG2a, IgG2b, and IgE, we found that IgM is absent on basophils, while the IgG subtypes and especially IgE is detectable in large amounts on the surface of basophils. The amount of IgE captured by basophils by far outweighs the amount of IgG on basophils. Binding of IgE was only marginal on other cell types. The virtual absence of the high-affinity IgG Fc receptors Fc\(\gamma\)RI and Fc\(\gamma\)RIII as well as the high-affinity receptor for IgE. In conjunction with our data, one now can conclude that basophils capture Ags by binding Ag-specific IgE molecules on high-affinity IgE receptors. The high-affinity IgG Fc receptor CD64 that was recently shown to be expressed on murine mast cell lines (P815, 10P2) and on APCs (dendritic cells, macrophages) has no major contribution to Ag binding on basophils.

How could basophils obtained from naive mice with IgE molecules already bound to their IgE receptors capture additional allophycocyanin-specific IgE molecules from the plasma of allophycocyanin-immunized mice? First, the IgE receptors on basophils from naive mice might not be completely occupied by the low concentrations of IgE (0.55 \(\mu g/ml\)) detected in the plasma of these mice. Second, the incubation of basophils with plasma from allophycocyanin-immunized mice containing much higher IgE concentrations (4.6 \(\mu g/ml\)) might result in an up-regulation of high-affinity IgE receptors and a decreased loss of surface Fc\(\gamma\)RI (24–26). It was shown that IgE molecules (5 \(\mu g/ml\)) induce an \(\sim 4\)-fold up-regulation of Fc\(\gamma\)RI expression within 3 h on murine mast cells (25). Up-regulation of high-affinity IgE receptors might explain the much higher binding of allophycocyanin, when basophils were incubated with plasma from allophycocyanin-immunized mice at 37°C compared with an incubation on ice. In addition, up-regulation of IgE receptors also appears as the most likely explanation why the blockade of potentially unoccupied IgE receptors with IgE control Abs did not prevent the subsequent capture of allophycocyanin-specific IgE Abs at 37°C. Since basophils were extensively washed after incubation with plasma, an interaction of allophycocyanin-specific IgE molecules with low-affinity receptors for IgE appears unlikely. The up-regulation of Fc\(\gamma\)RI by IgE molecules would enable basophils to bind newly added IgE Abs and always present a representative sample of plasma-derived IgE molecules on their surface despite the high-affinity interaction between IgE and Fc\(\gamma\)RI.

Basophils are important regulators of humoral immune responses. They influence B cell function by secretion of cytokines and also express CD40L that stimulates B cell proliferation (14, 30, 31). Via CD40L and the secretion of the cytokines IL-4, IL-6, and IL-13, basophils induce a Th2 deviation of the immune response and induce class switching to IgE and IgG4 in humans (14–16, 31, 32). The capture of Igs by basophils might be important during both the recognition phase and memory responses. In both phases, the amount of Ag is greater than the amount of specific Igs in the plasma and therefore free Ag is available for cross-linkage of surface Igs on basophils. At later time points, an excess of specific Ab would prevent binding of Ags to basophils and thereby exclude basophils from participating in immune regulation. The high expression of the chemokine receptor CCR2 that is also critical for migration of APCs (monocytes/macrophages and dendritic cells) may help to localize basophils to sites of Ag recognition and enhance their influence on humoral immune responses, especially in spleen and bone marrow.
In summary, we have identified Ag-capturing non-B cells as basophilic granulocytes that bind intact Ags in an IgE and IgG receptor-dependent manner. By their ability to capture Ags and respond to cross-linkage of surface IgGs, basophils may play an important role in supporting and accelerating humoral immune responses in the recognition phase and during a memory response.

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