

Antigen-driven basophil activation is indicative of early *Necator americanus* infection in IgE-seronegative patients

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Background: Parasitic worms induce a strong, polarized T_H2-type immune response. The kinetics of gastrointestinal nematode-induced T_H2-type responses, especially in the context of primary infection, have been extensively studied in experimental infection models but not in human subjects. **Objective:** We sought to determine the kinetics of basophil sensitization in subjects infected with *Necator americanus* during the first 12 weeks after infection.

Methods: Thirty nonasthmatic subjects with allergic rhinoconjunctivitis were randomized in a double-blind manner to cutaneous administration of either 10 hookworm infective larvae or histamine placebo. Blood samples were taken at regular intervals for 12 weeks, and basophil activation was determined in whole blood by measuring CD63 and CD203c levels on stimulation with *N. americanus* excretions/secretions. Parasite-specific immunoglobulin responses were assessed by means of ELISA and Western blotting.

Results: Median values reflecting basophil activation (CD203c/CD63 double-positive cells) in the excretion/secretion-stimulated infected group steadily increased after week 4, consistently achieving statistical significance compared with the placebo group between 6 and 12 weeks after infection. Only parasite-specific IgM levels increased significantly during this period, whereas total and parasite-specific IgE levels did not differ between groups.

Conclusion: Basophils are sensitized early in the context of a low-dose primary infection with *N. americanus* in the absence of measurable total and specific IgE serum level increase. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Helminth, hookworm, *Necator americanus*, basophil, basophil activation test

Abbreviation used

DPBS: Dulbecco PBS
E/S: Excretions/secretions
FITC: Fluorescein isothiocyanate
fMLP: N-formyl-methionyl-leucyl-phenylalanine
L3: Infective third larval stage
PE: Phycoerythrin

Nematode infections of human subjects and experimental animals are well known for inducing a T_H2 response, with associated eosinophilia and IgE serology.^{1,2} The kinetics of gastrointestinal nematode-induced T_H2-type responses, especially in the context of primary infection, have been extensively studied in experimental infection of animals but less so in human subjects. Such studies are usually anecdotal or involve relatively small numbers of volunteers and often lack appropriate control groups.

Nevertheless, the consensus of these studies is that experimental human infection with *Necator americanus* has to be repeated before a significant increase in parasite-specific IgE levels is seen (see Table E1 in this article's Online Repository at www.jacionline.org). The work presented here confirms that it is difficult to detect parasite-specific IgE serologically after experimental infection with 10 infective larvae. However, this single low-dose infection induces measurable sensitization of peripheral blood basophils. We describe a flow cytometric technique enabling reliable detection of progressive sensitization in hookworm-infected subjects and quantitation of basophil activation by using whole blood samples. The technique uses the combination of a basophil-specific surface activation marker (CD203c)^{3,4} and a degranulation marker (CD63),⁵ allowing detection of basophil degranulation in whole blood without having to recur to basophil purification.

We show that most IgE-seronegative patients infected with hookworms have basophil competence within 6 to 8 weeks after infection, coinciding with or after egg deposition, and specific serum IgM level increases in the absence of a measurable specific serum IgE level increase.

METHODS

Subjects and ethical approval

Subjects were recruited by means of local advertisements in the context of a randomized placebo-controlled double-blind clinical trial (ClinicalTrials.gov identifier NCT00232518). This study is described in more detail elsewhere.⁶ The study was reviewed and approved by the Nottingham Research Ethics Committee and the Research and Development Department at the Nottingham University Hospitals.

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Infection and placebo

The randomization was in blocks of 4 according to a computer-generated random code. Randomized subjects were infected transdermally with 10 *N americanus* larvae, as described previously.⁷ Subjects allocated to the placebo group were given a histamine dihydrochloride solution-containing patch (1.7 mg/mL; Diagenics Ltd, Milton Keynes, United Kingdom).

Blood stimulation and flow cytometric analysis

Blood was taken at weekly intervals initially, followed by biweekly sampling until week 12 after treatment. A stool sample collected within 24 hours of the corresponding visit was provided, and egg content was quantified as described elsewhere.⁷ One hundred-microliter aliquots of heparinized whole blood were incubated in FACS tubes containing different stimuli or controls in a water bath at 37°C for 10 minutes and then transferred to an ice bucket for another 10 minutes before staining.

Positive control stimuli were 10 μ M/L N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich, Gillingham, UK) or goat polyclonal anti-human IgE at 120 μ g/mL (Sigma-Aldrich). Negative controls were 10 μ L of Dulbecco PBS (DPBS; Cambrex, Verviers, Belgium) and an isotype control stimulated with 10 μ M/L fMLP. Basophil sensitization to hookworms was assessed by using 10 μ g/mL *N americanus* adult-stage larval excretions/secretions (E/S) produced as described previously⁸ or 10 μ g/mL recombinant hookworm calreticulin.⁹ The samples were incubated with 10 μ L of anti-CD63-fluorescein isothiocyanate (FITC; Immunotech, Marseille, France) and 10 μ L of anti-CD203c-phycoerythrin (PE; Immunotech) or anti-IgG1-FITC (Immunotech) and anti-IgG1-PE (BD Biosciences Pharmingen, San Jose, Calif) isotype controls and left in the dark at 4°C for 30 minutes. Erythrocytes were then lysed in the dark at room temperature for 10 minutes with 2 mL of lysis solution (PharmLyse, BD Biosciences Pharmingen). Cells were centrifuged at 600g for 6 minutes and washed once with 2 mL of lysis solution. The supernatant was discarded, and the cells were fixed in 0.5% formaldehyde/DPBS.

Because identification of basophils in whole blood relied solely on CD203c expression (in combination with side-scatter/forward-scatter gating), the incubation time was chosen as 10 minutes, which allows optimal detection of CD203c,^{10,11} rather than longer incubation times, which might have been more suitable for CD63 and have been shown by some authors to peak after 20 to 25 minutes.^{10,12}

The stained blood samples were measured on an EPICS Altra sorter (Coulter, High Wycombe, United Kingdom), and data were analyzed with WinMDI version 2.8 (The Scripps Research Institute, La Jolla, Calif). Dot plots were obtained by representation of side scatter versus forward scatter (see Fig E1, A, in this article's Online Repository at www.jacionline.org). By using the R1 gating, dot plots with CD203c-PE as the y-axis and CD63-FITC as the x-axis were used to determine the percentage of degranulated basophils in the upper right quadrant (defined as CD203c/CD63 double-positive cells, see Fig E2 in this article's Online Repository at www.jacionline.org). The percentage of gated cells was compared between the *N americanus* and placebo groups at each given time point by using the Mann-Whitney *U* test (SigmaStat 3.0 (Aspire Software International, Ashburn, NC)).

N americanus E/S-specific immunoglobulin isotypes and total IgE ELISAs

ELISA measurements of E/S-specific immunoglobulins and total IgE levels were performed as described previously¹³ and in the Methods section of this article's Online Repository at www.jacionline.org. Samples were compared with a standard curve derived from reference samples and relative E/S-specific immunoglobulin subclass concentrations and total IgE concentrations interpolated from a calculated sigmoidal dose response (variable slope) by using GraphPad Prism 3.0 (GraphPad Software, Inc, La Jolla, Calif). Data were analyzed with the unpaired Student *t* test.

Western blot analysis of anti-*N americanus* responses

N americanus infective third larval stage (L3) homogenate was used for detection of specific antibodies in plasma samples on Western blots, as described in detail in the Methods section of this article's Online Repository.

RT-PCR and quantitative RT-PCR analysis of cytokine mRNA expression

IL4 and *IL13* mRNA upregulation 1 hour after stimulation was assessed by means of RT-PCR and confirmed by means of quantitative RT-PCR with the primers and conditions described previously¹⁴ and in the Methods section in this article's Online Repository. Under these experimental conditions, IL-4 and IL-13 are mainly derived from basophils.¹⁵

RESULTS

In this study a flow cytometric technique is described that allows quantification of basophil degranulation in whole blood samples. The technique is based on the R1 gate shown in Fig E1, which includes most basophils but also monocytes and other leukocytes. As shown with highly enriched basophils, the gate will include both resting (R2; Fig E1, B) and activated (R3; Fig E1, C) basophils. Applying this gating to all samples, basophil activation can be quantified by calculating the percentage of CD63 and CD203c double-positive basophils before and after stimulation (see Fig E2).

We have used this assay to measure basophil sensitization at weeks 0, 1, 2, 3, 4, 6, 8, 10, and 12 after treatment in 30 subjects recruited for a double-blind randomized study. Three subjects withdrew from the study before completion. Of the remaining 27 participants, 13 had been infected with 10 *N americanus* L3, and 14 were given a histamine placebo. As shown in Fig 1, median values in the hookworm-infected group stimulated with E/S steadily increased after week 4, consistently achieving statistical significance ($P < .01$) compared with the noninfected group at weeks 6 ($P = .008$), 8 ($P = .006$), 10 ($P = .002$), and 12 ($P = .002$) after infection. Single incidences of apparently significant differences between the groups were also found at week 0 ($P = .040$) for the control stimulation and at weeks 4 ($P = .028$) and 12 ($P = .013$) after stimulation with fMLP. These are not part of a pattern, and the *P* values are larger than those obtained with E/S as a stimulus, suggesting that these differences have arisen by chance.

The median values in the placebo group stimulated with E/S did not show any increase over time. The stimulation with recombinant hookworm calreticulin did not show any sensitization in either group. Successful infection with hookworms was monitored by means of detection of eggs in stool samples (Fig 1, showing mean eggs per gram of feces $\pm$ SD) first seen 6 weeks after infection and a larval culture at termination of the study (Table I). This is in agreement with the expected prepatency of about 44 to 58 days (see Table E1). No eggs were detected in the placebo group.

No eggs were detected in 4 subjects from the hookworm group (subjects 4, 34, 49, and 50; Table I). In 3 of these egg-negative samples (subjects 4, 49, and 50), larval cultures were also negative, whereas infection could be confirmed by cultures in subject 34. The absence of eggs in repeated stool samples and larval cultures in the context of an infection with only 10 infective worms might be explained by the loss of worms during migration to the gut, the inability to establish a successful colonization of the gut, and/or single-sex infection.

In subjects 4, 49, and 50 establishment of basophil competence and an increase in eosinophil counts in peripheral blood suggested that infection had taken place despite the lack of detectable eggs. Subject 22 had strongly positive basophil test results throughout the study (from weeks 0-12), suggesting previous exposure to hookworms or other cross-reactive nematodes, and was therefore

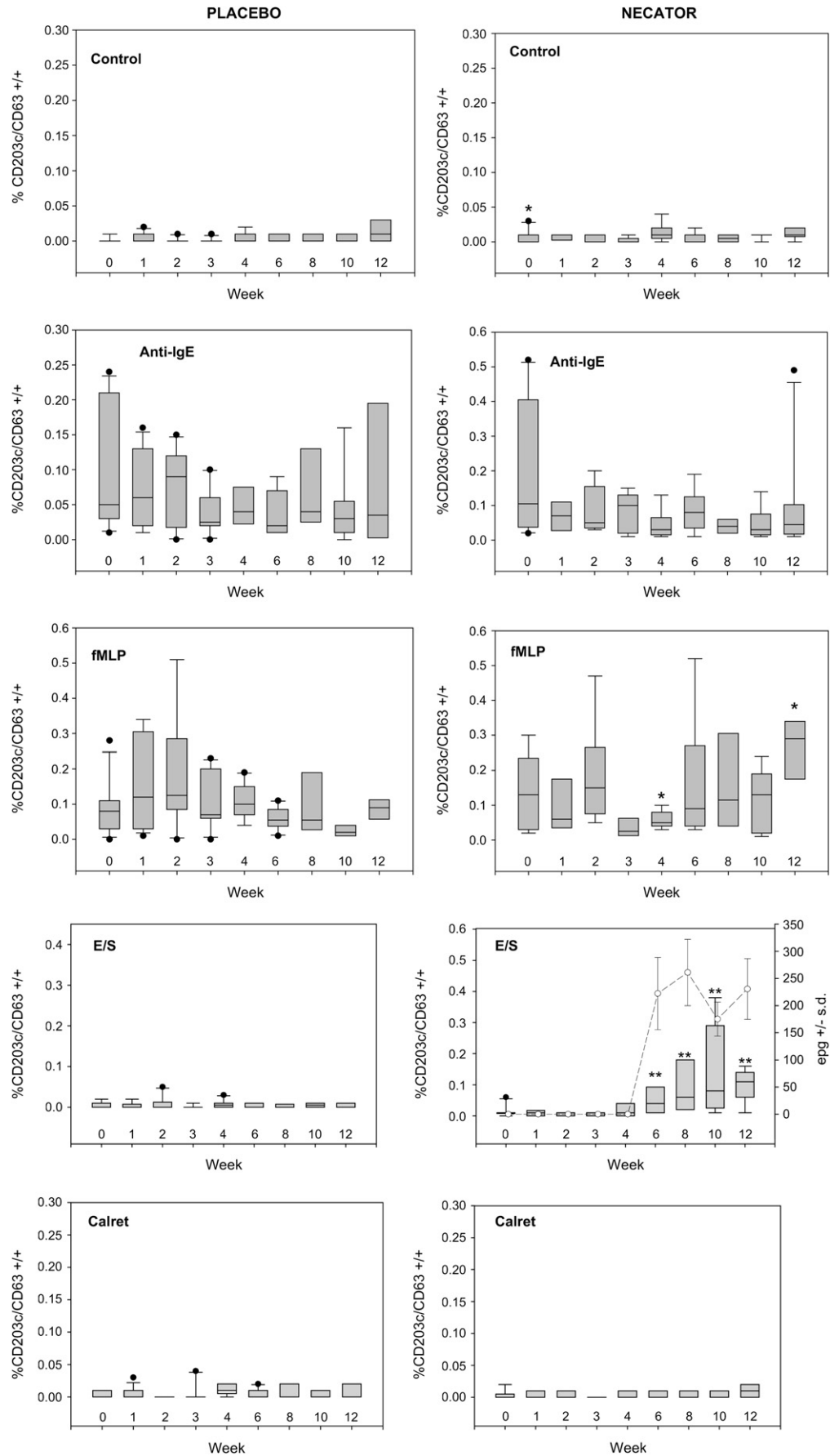


TABLE I. Time of first egg detection in hookworm-infected subjects and first time of appearance of basophil sensitization

Subject no.	2	4	10	12	15	20	22	28	29	34	44	49	50
Time of first egg detection (wk)	8	—	6	8	6	6	8	6	6	—	6	—	—
Larval culture (week 12)	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg
Basophil conversion (wk)	8	12	4	10	5	6	0	6	NA	—	8	8	6

No eggs were detected at any time point in the placebo group.

Pos, Positive; Neg, negative; NA, sample not available; —, no eggs detected.

excluded from the analysis. From Table I, it is clear that basophil sensitization usually occurs around the time of egg deposition (weeks 6–8). This is also the time of eosinophilic infiltration into the gut and corresponds to a peak in peripheral blood eosinophilia.⁷

N americanus-specific immunoglobulins of the IgM, IgG, and IgE isotypes and total IgE levels in plasma samples were also determined by means of ELISA (Fig 2, A). No increased antibodies were detected in the placebo group. In the hookworm group IgM levels increased significantly after 6 weeks, peaking at week 8. Specific IgG levels increased steadily from weeks 6 to 12 but did not achieve statistical significance. Neither specific nor total IgE levels showed an increase compared with those seen in the control group. Specific IgG levels in subjects 34, 44, and 49, in whom no parasite eggs had been detected, also confirmed successful infection. The Western blot shown in Fig 2, B, shows the IgG response to adult *N americanus* E/S in infected subjects.

Specific responses can be observed by using week 12 pooled *N americanus*-treated plasma (Fig 2, B, lane 3) and are comparable with the response found in natural field infections (Fig 2, B, lane 6: Papua New Guinea reference serum) but not found in week 12 pooled placebo-treated plasma (Fig 2, B, lane 5). Comparable results for IgM are shown in Fig 2, C, with marked immunoreactivity only seen in the *N americanus*-infected group at week 12 (Fig 2, c, lane 3) and the Papua New Guinea reference serum (Fig 2, c, lane 6). The ELISA and Western blot experiments both demonstrate that a single low-dose experimental infection of previously unexposed individuals results in an immunoglobulin response comparable with that seen in a natural infection, without the need for repeated infections.

Two years after primary infection, 5 infected subjects who had chosen not to take anthelmintics and remained infected after completion of the trial were assessed for basophil sensitization, specific IgE levels, and cytokine synthesis. All 5 subjects showed strong basophil reactivity when their blood was stimulated with E/S (see Fig E3 in this article's Online Repository at www.jacionline.org). To determine whether CD203c and CD63 upregulation on basophils correlated with the production of T_H2-type cytokines, *IL4* and *IL13* mRNA levels were determined by means of RT-PCR and quantitative RT-PCR in whole blood 1 hour after stimulation with E/S (Fig 3).¹⁶ In all 5 subjects, incubation with E/S led to an upregulation of mRNA for both cytokines, suggesting that CD63 and CD203c induction is a reflection of anaphylactic-type degranulation involving *de novo* T_H2 cytokine synthesis. Overall, specific IgE levels were not significantly increased when

compared with those at week 0, with only subject 15 showing a statistically significant increase ($P = .0069$, *t* test; data not shown). Thus the picture obtained 2 years after infection is similar to what was seen after 12 weeks, with basophil sensitization (and antigen-dependent induction of IL-4 and IL-13 cytokine synthesis) in the absence of detectable levels of parasite-specific IgE in serum.

Altogether, our findings confirm that specific IgE levels are not measurable serologically in a primary, low-dose infection with *N americanus*, which is in good agreement with previous self-infection studies (summarized in Table E1). However, we show for the first time that despite the absence of measurable levels of specific IgE in plasma, basophils become sensitized around the time of egg deposition and can be activated by *in vitro* exposure to the parasite's secretions.

Two years after infection, basophils were still sensitized to parasitic antigens and upregulated T_H2 cytokine mRNA on antigenic stimulation. Although this study was limited to the measurement of basophil activation markers and did not include a full assessment of mediator and cytokine release, we believe that this event, given the ability of activated basophils to secrete the appropriate cytokine cluster, might be pivotal for the establishment of the T_H2 milieu.¹⁷

DISCUSSION

This study shows that in low-dose, single primary infections with *N americanus*, basophils become sensitized in the absence of measurable parasite-specific IgE levels in the serum. Several recent studies have used either CD203c or CD63 for detection of *in vitro* basophil activation by allergens such as insect venom,¹⁸ Fel d 1,¹⁹ or latex.²⁰ These and other studies have compared their relative usefulness for detection of basophil activation. The controversial issue of whether CD63 or CD203c is the more reliable indicator of basophil activation has been discussed, and the relative arguments have been highlighted in several recent reviews.^{21,22} Surprisingly, the combination of both markers has only been used in a few studies.^{23–25} The most recent study suggests that CD203c is a more sensitive marker than CD63 for fMLP but not anti-IgE-stimulated basophils.²³ Our study uses the combination of both antigens, without the necessity to include other markers for basophil identification. CD203c enables specific and unequivocal identification of basophils in whole blood, whereas CD63, which is normally not found on resting basophils, enables quantification of basophil degranulation. The combination enables an easy measurement of basophil activation by using

FIG 1. Percentage of CD203c/CD63 double-positive (+/+) basophils in whole blood of placebo-treated ($n = 14$, left) versus *N americanus*-treated ($n = 12$, right) subjects n weeks after infection. * $P \leq .05$ and ** $P \leq 0.01$ (Mann-Whitney *U* test), comparing the placebo-treated versus *N americanus*-treated groups for each time point. Superimposed on the E/S plot are the egg counts (circles; eggs per gram of stool sample expressed as means $\pm$ SDs; $n = 9$).

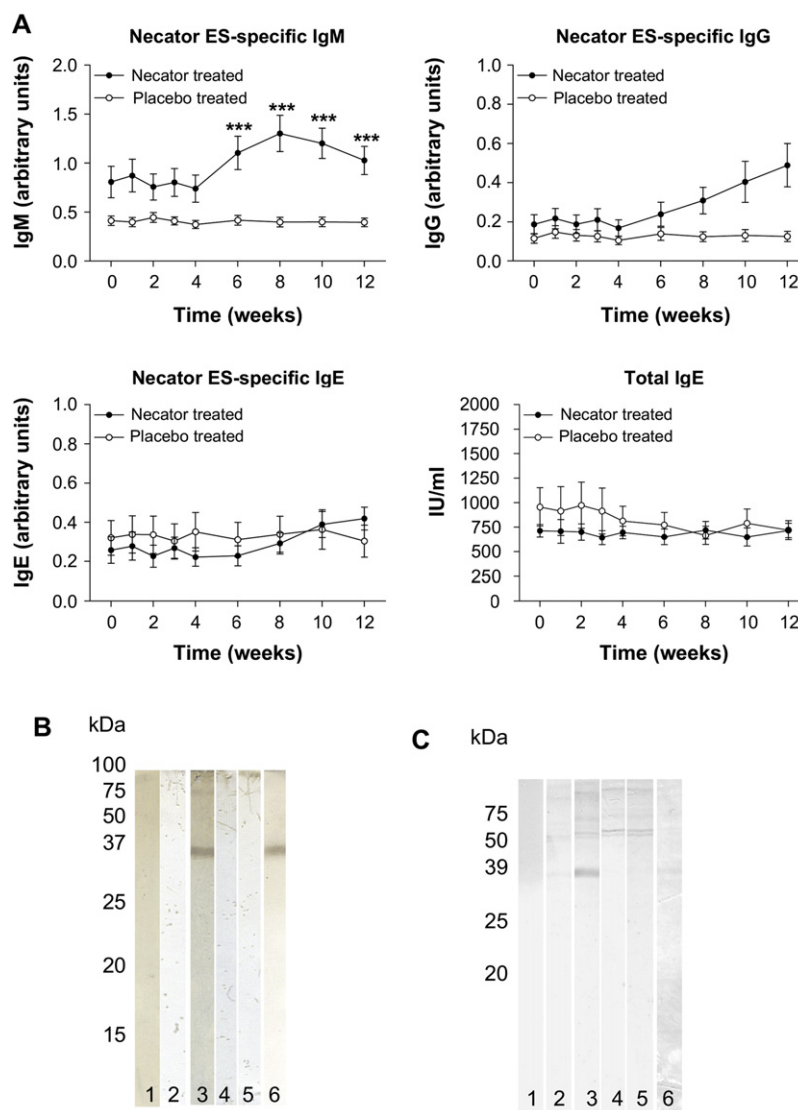


FIG 2. *A*, *N. americanus* E/S-specific immunoglobulins (AU $\pm$ SDs). *** $P < .001$, unpaired t test comparing groups at individual time points. **B** and **C**, Western blot of anti-*N. americanus* IgG (**Fig 2, B**) and IgM (**Fig 2, C**). Lane 1, Negative control; lanes 2 and 3, *N. americanus*-treated group (week 0 and week 12); lanes 4 and 5, placebo group (week 0 and week 12); lane 6, PNG reference serum.¹²

small volumes of unprocessed whole blood and is therefore very suitable for the large number of samples collected in this study. The combination also helps avoid the pitfalls of using either CD63¹¹ or CD203c as sole indicators of activation. CD203c basal levels are known to vary greatly between subjects and depend largely on IL-3,²⁵ the main priming factor for basophils, levels of which are increased during inflammatory processes. CD63 is not specific for basophils, making it necessary to identify basophils through other surface markers, such as Fc ϵ RI, which can also be found on other leukocytes present in whole blood, such as monocytes,²⁶ eosinophils,²⁷ and neutrophils.²⁸ Because of the low levels of basal expression of CD203c in several subjects in the absence of basophil activation (eg, in the placebo group or in the infected group before week 6), it was not possible to calculate the number of activated basophils as a percentage of total basophils. This would have required the inclusion of additional markers for identification of basophils (eg, chemokine receptor

3 [CCR3]²⁹ or chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes [CRTH2]³⁰). Also, determination of the mean fluorescence intensity for CD203c as an alternative parameter did not increase the sensitivity of detection (data not shown). Thus using the number of double-positive CD63/CD203c cells was the only quantification method that allowed us to reliably discriminate the infected from the noninfected subjects at week 12, with an accuracy of 87%. We did not find a shift in baseline CD203c expression in the infected group.

We believe that a similar protocol based on the simultaneous detection of CD63 and CD203c could be used for the detection of sensitization of basophils to common allergens. Such a protocol would overcome many of the limitations encountered when using CD63 or CD203c in isolation.

Studies looking at the kinetics of basophil competence establishment in a cohort of immunologically naive subjects infected with helminths have not been possible before because the use of

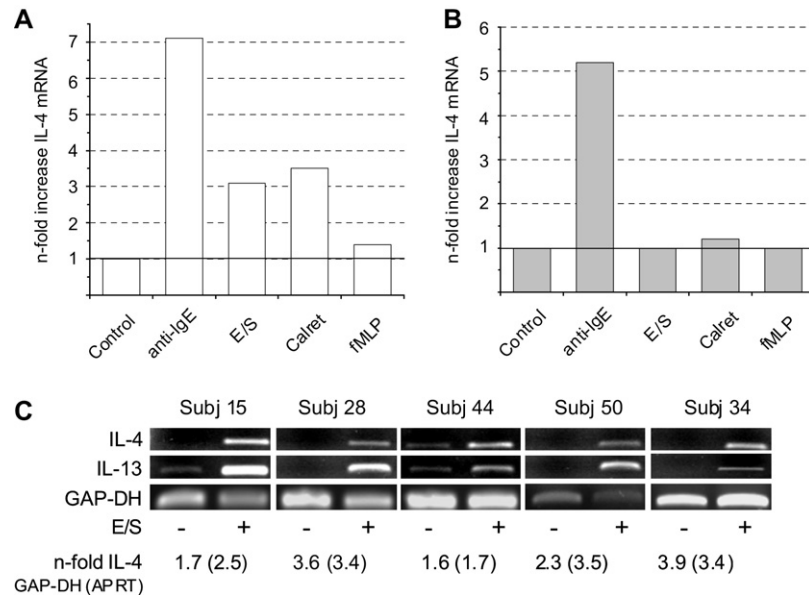


FIG 3. RT-PCR analysis of *IL4* and *IL13* mRNA production in blood of subjects 15, 28, 44, 50, and 34 two years after infection with *N americanus*. Stimulation with E/S results in upregulation of *IL4* and *IL13* mRNA 1 hour after stimulation. The values for relative n-fold mRNA increase in E/S-treated versus untreated blood were determined by using SYBR Green quantitative RT-PCR in triplicates with glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) and adenine-phosphoribosyltransferase (APRT) as reference genes, calculated by using the Pfaffl equation¹⁶ and the experimentally determined efficiencies for each primer pair.

markers, such as CD63 and particularly CD203c, for the monitoring of basophil activation is a relatively recent development. Thus our study is the first to describe the occurrence of basophil sensitization in the absence of measurable specific IgE levels in serum, coinciding with parasite maturation and egg deposition. The results reveal a clear pattern of increasing basophil sensitization, with highly significant *P* values beginning at week 6, which is only seen in the *N americanus*-treated group.

Our results confirm the findings of previous smaller-scale studies (see Table E1). In a self-infection study by Ball and Bartlett,³¹ "reagins" were demonstrated 4 weeks after infection by using the Prausnitz-Küstner skin test.³² This result suggests that parasite-specific IgE was present in the infected individual's serum. In our study 1 infected subject displayed basophil sensitization 4 weeks after infection, whereas most subjects converted after 6 weeks. In a second self-infection report by Ogilvie et al,³³ however, increased specific serum IgE levels were only detected after the third and fourth repeated infection with 250 L3 specimens. In the study by Maxwell et al,³⁴ a single primary hookworm infection with 50 infective larvae produced clear signs of transient gastrointestinal morbidity and pronounced peripheral blood eosinophilia but no measurable T-cell and B-cell immune responses. Small increases in anti-*N americanus* IgE and IgG levels were only noted in some subjects. In the most recent self-infection study by Wright and Bickle,³⁵ specific IgE levels were increased approximately 7-fold 811 days after primary infection and again doubled 3 months after secondary infection.

These results suggest that in primary low-level infection a measurable increase in specific IgE levels occurs only after prolonged patency. There are no doubts, however, that repeated infection in the tropics will lead to high levels of both specific and total IgE.^{36,37} Our findings show that basophil sensitization occurs as early as 4 to 6 weeks after infection in a low-dose primary infection. The observation that parasite-specific IgM levels

increase after 6 weeks, coinciding with the first appearance of basophil sensitization in the absence of specific IgE, suggests the possibility of an alternative mechanism of basophil activation involving formation of IgM immune complexes and subsequent complement activation. However, when the sera obtained 2 years after infection were used for resensitization of stripped basophils from a noninfected donor, none of the sera tested was able to sensitize the basophils, with the exception of subject 28, whose serum had measurable specific IgE levels (see Fig E4 in this article's Online Repository at www.jacionline.org). These results provide a separate confirmation of the ELISA data, which did not detect a significant increase of specific IgE in the same samples.

We believe that this early sensitization of basophils is an important step in a positive antigen- and basophil-driven amplification cascade that finally results in the full T_H2 -type response typical of helminthic infection.

As shown recently, basophils are the major source of antigen-driven IL-4 in human filarial infections.³⁸ On a cell-to-cell ratio, basophils can produce greater quantities of IL-4 than $CD4^+$ T cells in response to antigens.³⁹⁻⁴¹ There is also increasing evidence for a pivotal role of basophils during early nematode infection coming from animal experimental infection models. Min et al⁴² tracked the source of IL-4 in G4 transgenic mice infected with *Nippostrongylus brasiliensis* to basophils. IL-4-producing basophils were found in the lung and unexpectedly in the liver. On restimulation with parasitic antigens, basophils from the livers of the infected animals were the main IL-4-producing cell population. Also, our previous work has shown that human basophils express a type 2 cytokine profile when exposed to *N americanus* secretions *in vitro*, despite the absence of IgE.⁴³ This was shown to be dependent on intact proteolytic activity of the secretions. In this study proteolytic activity of the secretions was probably inhibited by the high concentrations of protease inhibitors present in blood and thus did not activate the basophils of naive subjects

by means of IgE-independent pathways or interfere with the assessment of basophil sensitization. The situation in peripheral blood, however, might not reflect the interaction in the gut tissue, such as with resident mast cells. To demonstrate that the basophil activation described in this study is indeed IgE mediated, the use of the IgE surface-stripping techniques, as described by Pruzansky and Patterson,⁴⁴ would have been useful; however, the use of this technique was not possible because of the small volumes of blood available for basophil studies (1-2 mL).

Altogether, our findings suggest that basophils are sensitized early in the course of a primary infection, even in the absence of measurable specific IgE level increases in serum, and could initiate an early T_H2 switch in response to helminthic infections. Whether this early cytokine induction is mediated by receptor-bound IgE remains to be shown.

Interestingly, Mitre and Nutman⁴⁵ showed that basophils are still responsive to filarial antigens years after antifilarial treatment in the absence of active infection or reinfection. This is possibly due to the survival of adult parasites in patients treated with diethylcarbamazine, the persistence of antigenic depots in soft tissues, or both. The significance of this finding is that on reinfection, subjects with previous exposure might rapidly reacquire a T_H2 -skewed immune response by means of antigenic stimulation of sensitized basophils, a phenotype that, depending on the parasite considered, will affect reinfection rate. Ganguly et al³⁶ reported a rapid decrease in anti-hookworm IgE levels on treatment. In the context of the importance of parasite-specific IgE and the T_H2 phenotype in reinfection with hookworms,^{13,37} it will be interesting to assess whether hookworm infection, as seen with filariae, also leaves behind an "immunologic legacy" in the form of a basophil-bound IgE memory years after treatment.

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Clinical implications: Specific IgE is undetectable serologically during primary infection with *N. americanus*. However, basophil activation after exposure to hookworm antigens indicates early sensitization. Our study is the first to describe the appearance of basophil sensitization in the absence of measurable specific IgE levels in serum.

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METHODS

***N. americanus* E/S-specific immunoglobulin isotypes and total IgE ELISAs**

Ninety-six-well immunoassay plates (Nunc, Roskilde, Denmark) were coated with 50 μ L of *N. americanus* E/S at 2.5 μ g/mL in 50 mmol/L carbonate/bicarbonate buffer, pH 9.6, and incubated at 4°C overnight. For determination of total IgE levels, ELISA plates were coated with 2 μ g/mL anti-human IgE (BD Biosciences PharMingen). Plates were emptied and patted dry and blocked with 200 μ L of 1% BSA (Sigma-Aldrich, UK) in PBS for 1 hour at room temperature. Plates were washed 3 times with 300 μ L of PBS plus 0.05% Tween 20 (PBS/Tween) per well, and 50- μ L samples diluted with 1% BSA in PBS/Tween were added to relevant wells and incubated overnight at 4°C. Plates were washed 3 times with 300 μ L of PBS/Tween. 50 μ L of anti-human IgG peroxidase (1:1000; Binding Site, Birmingham, United Kingdom), anti-human IgM peroxidase (1:1000, Binding Site), or biotinylated anti-human IgE (1:500; BD Biosciences PharMingen, Oxford, United Kingdom) diluted in 1% BSA/PBS/Tween added per well and incubated at room temperature for 2 hours. At this point, total and specific IgE plates were washed 3 times with 300 μ L of PBS/Tween per well, and 50 μ L of streptavidin-peroxidase (BD Biosciences PharMingen) diluted 1:1000 in 1% BSA/PBS/Tween was added per well and incubated at room temperature for 30 minutes. Plates were washed 3 times with 300 μ L of PBS/Tween and 100 μ L of tetramethyl benzidine (Sigma-Aldrich, UK) substrate in 0.1 mol/L sodium acetate, pH 6.0, added per well and incubated at room temperature for 2 to 20 minutes until color developed. Color development was arrested by adding 15 μ L of 2 mol/L sulfuric acid per well, and plates were read at 450 nm in a spectrophotometric 96-well plate reader (MRX; Dynex Technologies, Chantilly, Va). Samples were compared with a standard curve derived from reference samples, and relative E/S-specific immunoglobulin subclass concentrations and total IgE concentrations were interpolated from a calculated sigmoidal dose response (variable slope) by using GraphPad Prism 3.0. Data were analyzed with the unpaired Student *t* test.

RT-PCR and quantitative RT-PCR analysis of cytokine mRNA expression

Sixty minutes after stimulation with anti-IgE or *N. americanus* E/S at 37°C, 250 μ L of heparinized whole blood (obtained from the five 2-year follow-up subjects, nos. 15, 28, 34, 44, and 50) was treated with 2 mL of PaxGene stabilization reagent (PreAnalytix), and total RNA was extracted with the PaxGene Blood RNA system (PreAnalytix), as directed by the manufacturer. cDNA was synthesized by using the GeneAmp RNA PCR core kit (Applied Biosystems, Foster City, Calif) with oligo-dT for priming. Conventional PCR and SYBR Green (iQ SybrGreen, Biorad, Hemel Hempstead, UK) quantitative PCR were performed by using the primers and conditions described previously.^{E1} Increases in gene expression were quantified as relative increase in treated versus control samples by using the Pfaffl equation and experimentally determined primer efficiencies.

Isolation of basophil-enriched PBMCs

Peripheral blood cells were isolated from 25 mL of heparinized blood by using a Ficoll-Hypaque/Percoll 100:6 mixture (vol/vol) with a density of 1.080 g/mL. The higher density increases the yield of basophils retained at the interphase. Basophil-enriched PBMCs were then washed twice in DPBS (without Ca²⁺/Mg²⁺), and the buffer was carefully and completely removed after the final centrifugation step (400g for 5 minutes) in separate FACS tubes.

IgE stripping and resensitization

Basophil-enriched PBMCs were incubated with 200 μ L of 10 mmol/L lactic acid buffer, 130 mmol/L NaCl, and 50 mmol/L KCl, pH 3.9, for 3 minutes on ice, with cells briefly resuspended after 1 and 2 minutes. The acid buffer was then neutralized with 2 mL of DPBS (without Ca²⁺/Mg²⁺) with 0.5% BSA and 0.1 mmol/L ethylenediamine tetraacetic acid, and cells were quickly separated from the supernatants by mean of centrifugation, followed by one more wash in the above buffer and final resuspension in 100 μ L of cell culture medium (RPMI 1640 and 10% FCS with additives). A proportion of cells was not stripped as a control for the donor's reactivity. Stripped cell suspensions were either incubated with sera from infected patients for 90 minutes at 37°C in a humidified cell incubator or left in cell culture medium without added serum. Cells were then stimulated with 1 μ mol/L fMLP, anti-IgE, or *N. americanus* E/S, as described in the Methods section, and basophil activation was monitored by means of flow cytometry with anti-CD63-FITC and anti-CD203c-PE.

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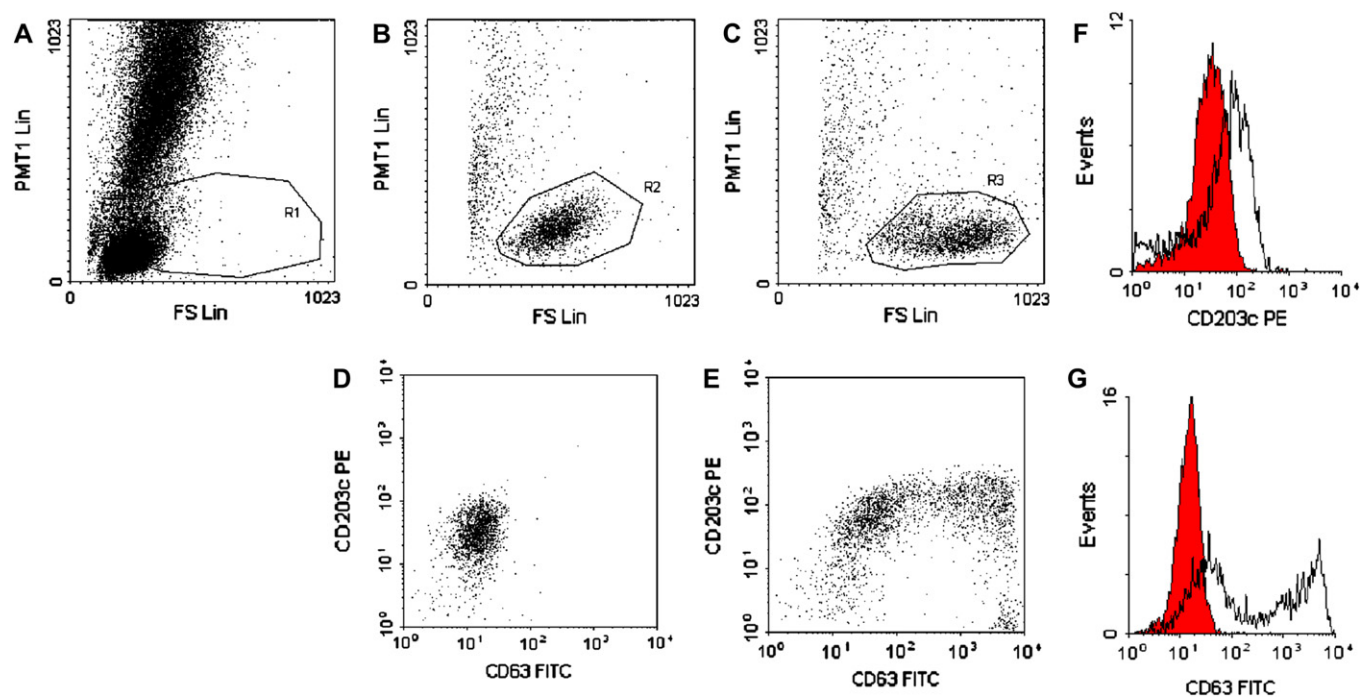


FIG E1. Gating used to detect whole blood basophil activation. **A**, Side scatter (*SS*, *Pmt1Lin*) versus forward scatter (*FS*, *FSLin*) of whole blood. **B** and **C**, Side-scatter versus forward-scatter dot plots of highly enriched basophils (>90% purity) before (Fig E1, *B*) and after (Fig E1, *C*) anti-IgE stimulation. **D** and **F** and **E** and **G** correspond to unstimulated (Fig E1, *B*) and stimulated (Fig E1, *C*) basophils, respectively, and illustrate CD203c and CD63 upregulation on activation.

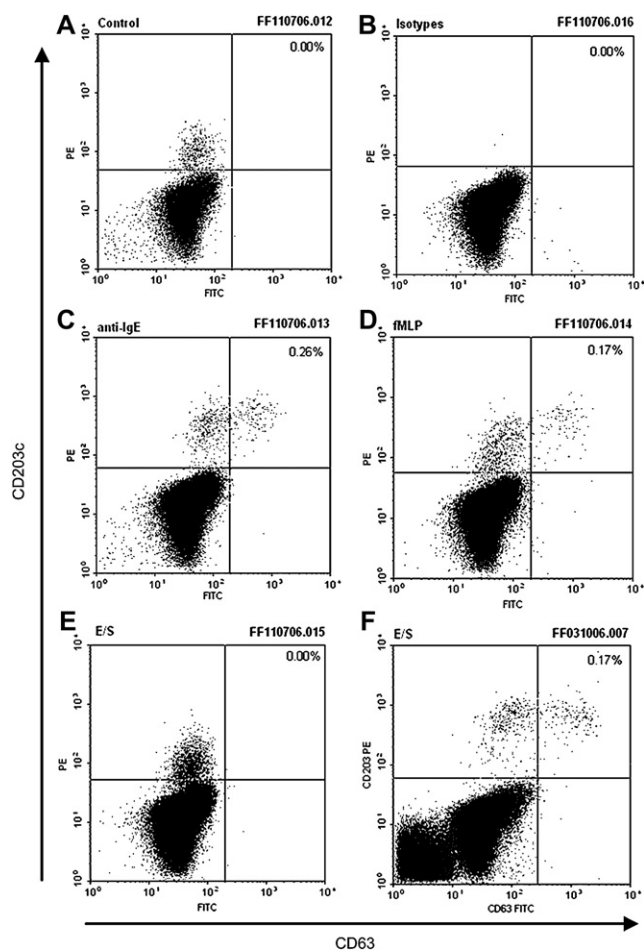


FIG E2. Examples of dot plots (y-axis, CD203c-PE; x-axis, CD63-FITC) of 100 μ L of whole blood stimulated with positive controls (C, anti-IgE; D, fMLP 10 μ mol/L), negative controls (A, buffer; B, isotype control), or 10 μ g/mL *N. americanus* E/S (E, negative subject; F, positive [infected] subject). Percentages in the upper right quadrant are double-positive CD203c/CD63 basophils based on the initial gating (R1 in Fig E1). The intra-assay (tube-to-tube) variation with this assay was $10\% \pm 5.99\%$ (mean $\pm$ SD, n = 4).

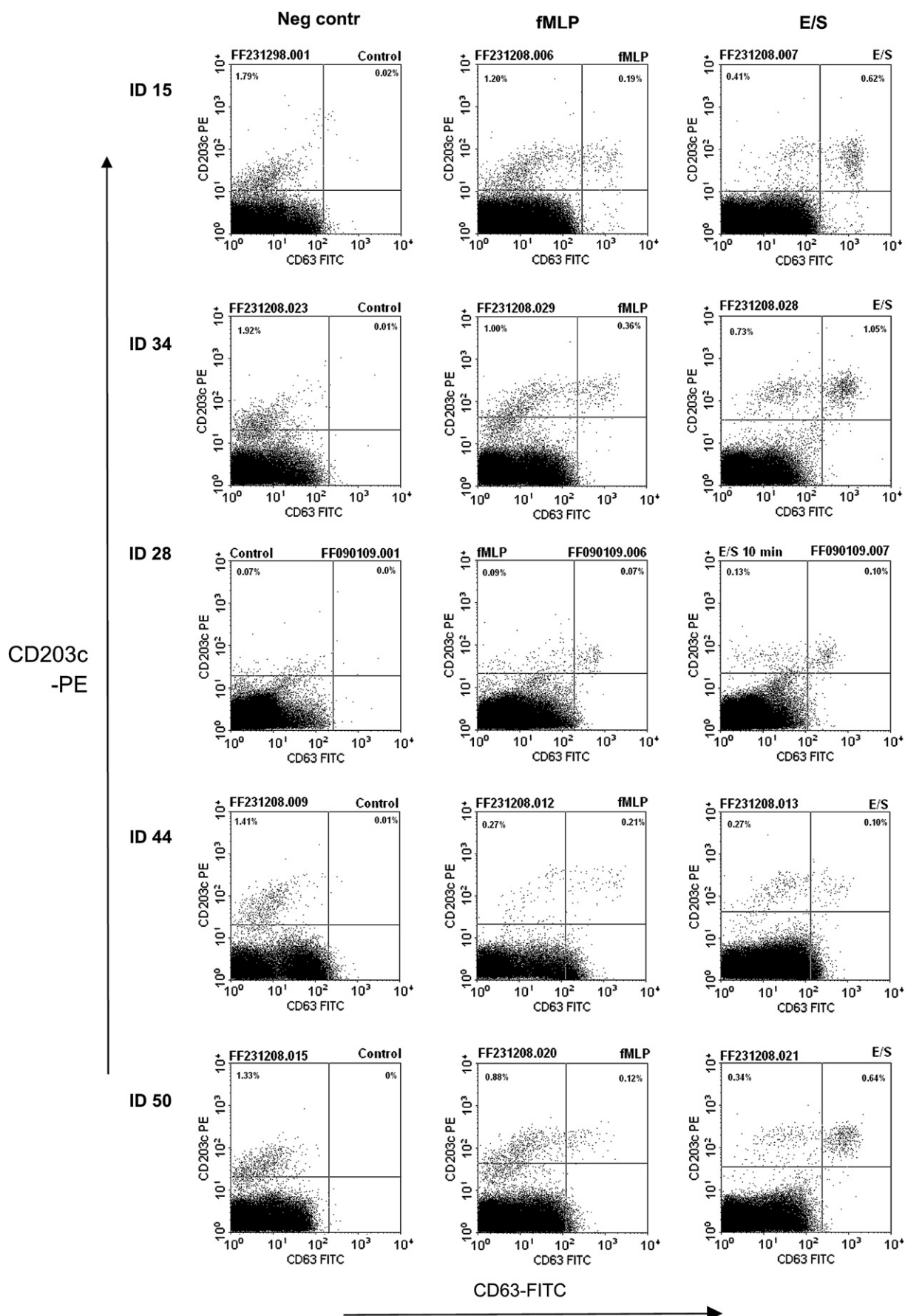
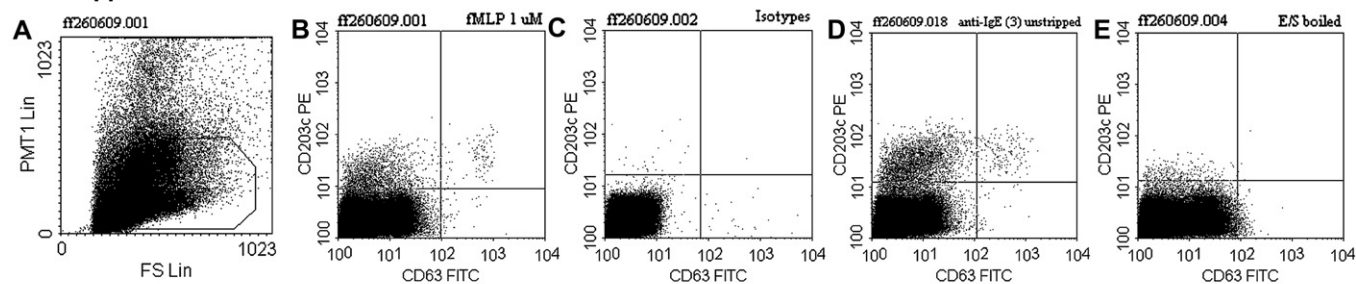




FIG E3. FACS analysis showing basophil sensitization in 5 infected subjects 2 years after infection. One hundred microliters of whole blood was stimulated with medium alone (control), 10 μ mol/L fMLP (positive control), or 10 μ g/mL *N americanus* E/S. Percentages in the upper right quadrant are double-positive CD203c/CD63 basophils based on the initial gating (R1 in [Fig E1](#)).

Unstripped PBMCs from naïve donor



Stripped PBMCs, not resensitized

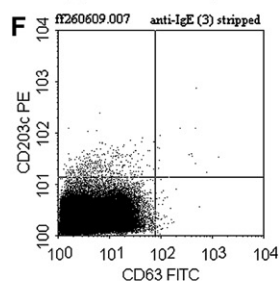
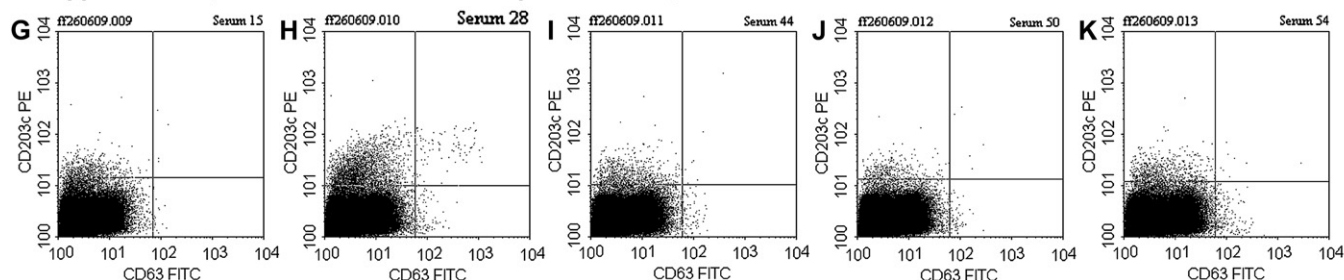
Stripped PBMCs, resensitized with infected patients' sera, simulated with *N. a.* E/S

FIG E4. Basophil-enriched PBMCs were stripped with lactic acid to remove surface-bound IgE from the basophils, as described. **A** shows a forward vs. side scatter dot plot of unstripped PBMCs, and the gate used for all other panels. Basophils responded to fMLP (**B**) or anti-IgE (**D**) with upregulation of CD203c and CD63 but not to heat-inactivated *N. americanus* E/S (**E**). Isotype controls (**C**) show lack of unspecific binding. After IgE stripping, basophils did not respond to anti-IgE stimulation (**F**), suggesting that surface IgE had been efficiently removed. Incubation with serum 28 (**H**) but not with the other 4 sera (Fig E4, **H**; **I-K**) was able to passively sensitize the donors' basophils. This finding is in agreement with the ELISA data, which did not detect a significant increase of parasite-specific IgE in the sera, with the exception of subject 28's serum.

TABLE E1. Synopsis of published self-infection studies with *N americanus* infective larvae with respect to specific IgE levels, infection protocols, and resulting prepatency times

Author	No. of subjects (control subjects)	Level of infection (no. L3)	Reinfection (no. L3)	Time point of specific IgE measurement	Method of IgE detection	Time of egg detection (d) pi	Result
Ball and Bartlett, 1969 ^{E2}	1 (100)	300	100 (2×) 25 (6×)	4 wk; 3, 6, and 12 mo	Prausnitz-Küstner test	NA	Positive after 4 wk, peak 3 mo pi
Ogilvie et al, 1978 ^{E3}	1 (0)	250	250 (3×)	Several	RAST	44-51	Significant increase of specific IgE only after third and fourth infection*
Cline et al, 1984 ^{E4}	21 (9)	75	—	ND	ND	56	ND
White et al, 1986 ^{E5}	3 (0)	50	—	ND	ND	ND	ND
Maxwell et al, 1987 ^{E6}	5 (0)	50	—	2 m pi	RIA	48-58	Small increases of specific IgE in some volunteers; no overall increase*
Ganguly et al, 1988 ^{E7}	30 (47)	Natural	Unknown	NA	REIA	NA	Marked increase
Wright and Bickle, 2005 ^{E8}	1 (0)	50	50	Day 811 after primary infection; 3 mo after reinfection	ELISA	53	715% increase above baseline; 94% increase above presecondary levels*

pi, After infection.

*The result is mentioned in the text, but corresponding data are not shown.