

Detection of allergen-induced genes in peripheral blood mononuclear cells of patients with allergic asthma using subtractive hybridization

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When stimulated with mite antigens, peripheral blood mononuclear cells obtained from patients with mite allergy release eosinophil chemotactic factor, a type of protein. Complementary DNA libraries were formulated from both peripheral blood mononuclear cells stimulated with mite antigens and those not stimulated with mite antigens and underwent subtraction and differential screening. The authors obtained 13 genes for which expression had increased because of mite antigens. Of these, nine were mitochondrial genes; two, h-satellite DNA III; one, factor XIII; and one, ferritin heavy subunit. As a result of polymerase chain reaction on both cDNA libraries, IL-5 genes were amplified only from the cDNA library that had been stimulated with mite antigens. (J ALLERGY CLIN IMMUNOL 1995;96:1076-82.)

Key words: Asthma, eosinophil chemotactic activity, subtractive hybridization, IL-5, lone-linker PCR, peripheral blood mononuclear cells, mite antigen, ferritin

Eosinophil accumulation is a prominent feature of allergic inflammatory disorders, such as allergic asthma, rhinitis, and atopic dermatitis. Although mechanisms that lead to specific eosinophil accumulation involve a complex interplay of multiple molecules and pathways, eosinophil response to certain chemotactic agents may be one potential mechanism. Because an influx of eosinophils into the skin,¹ nose,² or bronchi^{3,4} occurs within 6 to 12 hours after allergen provocation, mast cell-derived mediators were initially believed to function as the predominant eosinophil chemoattractant mechanism. However, with increasing evidence that allergen-induced eosinophil infiltration in chronic-phase or ongoing tissue eosinophilia is closely associated with T-cell activation status in the tissue,^{1,5,6} attention has been focused on T-cell

Abbreviations used

ECA:	Eosinophil chemotactic activity
GM-CSF:	Granulocytes-macrophage colony-stimulating factor
PBMCs:	Peripheral blood mononuclear cells
PCR:	Polymerase chain reaction

products. In fact, it has been shown that a variety of cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, and IL-5, are chemotactic for eosinophils.^{7,8}

In a previous study we reported that peripheral blood mononuclear cells (PBMCs) from patients with asthma who are sensitive to mite antigen extract produce eosinophil chemotactic activity (ECA) with the same antigen stimulation.⁹ Activity was unchanged by heating at 56° C for 30 minutes but was inactivated by heating at 100° C for 10 minutes. The molecular weight of this eosinophil chemotactic substance was greater than 30 kd as determined by an ultrafiltration study. Furthermore, the ECA was not neutralized with antibodies against GM-CSF, IL-3, or IL-5, suggesting that unknown lymphocyte products may mediate the ECA.

When these substances are purified from the

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mite-stimulated PBMCs, taking advantage of the ECAs, approximately 200 L of peripheral blood from subjects with mite allergy is required. Furthermore, it is difficult to isolate active substances. Therefore we have attempted to isolate ECA genes. However, we came across two problems. First, because the amount of PBMCs obtained from subjects with mite allergy is still limited, a complementary DNA library of good quality cannot be formulated with ordinary available methods; and second, no other information is available regarding the physicochemical properties of ECA genes, except that they should be proteins. To solve the first problem, we adopted a lone-linker polymerase chain reaction (PCR) technique,¹⁰ which enables cDNA amplification from a small amount of messenger RNA. To solve the second problem, we decided to use the so-called subtraction method, which subtracts the total cDNA of the nonstimulated PBMCs from the total cDNA of PBMCs stimulated with mite antigen extracts. This decision was made because ECA was observed only in the supernatant from the mite-stimulated PBMCs.⁹

METHODS

Isolation of mononuclear cell populations

Sixty milliliters of venous blood was collected in heparin from a patient with asthma who was sensitive to mite antigen extracts. The mononuclear cells were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation; isolated cells were washed three times and resuspended in a final concentration of 1×10^6 cells/ml in complete medium: alpha modification of Eagle's medium (Flow Laboratories, USA), 10% heat-inactivated fetal bovine serum (Flow Laboratories), supplemented with 100 IU/ml penicillin (Meiji Seika, Tokyo, Japan) and 100 µg/ml streptomycin (Meiji Seika).

Antigen stimulation

The mononuclear cells were stimulated for 72 hours with 0.1 µg/ml mite antigen extract (Torii Pharmaceutical Co., Tokyo, Japan) in a humidified atmosphere containing 5% CO₂ (mite-positive PBMCs). These antigen concentrations and periods proved to be optimal for inducing ECA production. As a negative control, cells were cultured in medium alone (mite-negative PBMCs).

Formulation of cDNA libraries with mite-positive and mite-negative PBMCs

After mite-positive and mite-negative PBMCs were frozen with liquid nitrogen, total RNA was extracted from approximately 3×10^6 cells of each set of PBMCs, with the ISOGEN kit (Nippon Gene Co., Toyama,

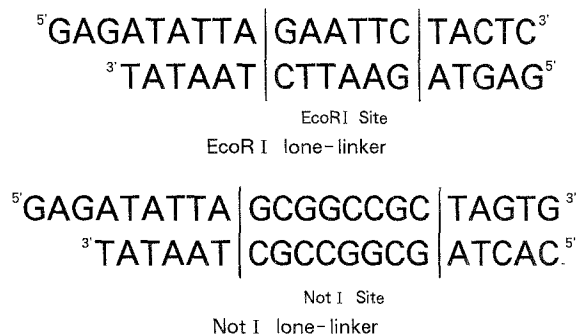


FIG. 1. Lone-linker structures used in this experiment.

Japan). From the total RNA thus obtained, cDNA was synthesized with the cDNA Synthesis System Plus RPN 1256Y/Z Kit (Amersham International Ltd., Little Chalfont, Bucks, U.K.). Mite-negative cDNA thus formulated was ligated with *NotI* lone-linker (Fig. 1), and mite-positive cDNA was ligated with *EcoRI* lone-linker (Fig. 1). After ligation, mite-negative cDNA underwent *NotI* digestion, and mite-positive cDNA underwent *EcoRI* digestion. Electrophoresis was performed on each digest with 1.0% low melting point agarose gel, and cDNAs ranging in size from 100 to 5000 bp were collected. Each cDNA then underwent 30 cycles of PCR, with the top strand of the added lone linker as the forward primer and the bottom strand as the reverse primer. Each amplified cDNA was collected by means of ethanol precipitation, and the cDNA library was made, although it was not inserted into a vector.

Subtractive hybridization

The mite-negative cDNA library was biotinylated with Photobiotin (Sigma Chemical Co., St. Louis, Mo.) according to the method of Forster et al.¹¹ Ten micrograms of the biotinylated mite-negative cDNA library and 1 µg of the mite-positive cDNA library were dissolved in 20 µl of subtractive hybridization buffer (50 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [pH 7.6], 0.2% sodium dodecylsulfate, 0.4 mmol/L ethylenediaminetetraacetic acid, 0.5 mol/L NaCl). Four capillaries, each containing 5 µl of this solution, were prepared; and both ends of the capillaries were flame-sealed. They were placed over aluminum blocks, which had been heated to 100° C, then allowed to stand for 5 minutes. Later, the temperature was slowly lowered to 65° C, then maintained at this temperature for 20 hours.

After hybridization, the solution was treated with streptavidin (Gibco BRL, Grand Island, N.Y.) according to the method of Dugid and Dinauer¹² and underwent phenol extraction to remove the biotinylated mite-negative cDNA, as well as the mite-positive cDNA, which had formed a hybrid with the biotinylated mite-negative cDNA. The remaining cDNA underwent 10 cycles of PCR with *EcoRI* lone-linker primers, and the amplified cDNA was inserted into the *EcoRI* site of λ-ZAPII (Stratagene, La Jolla, Calif.) to

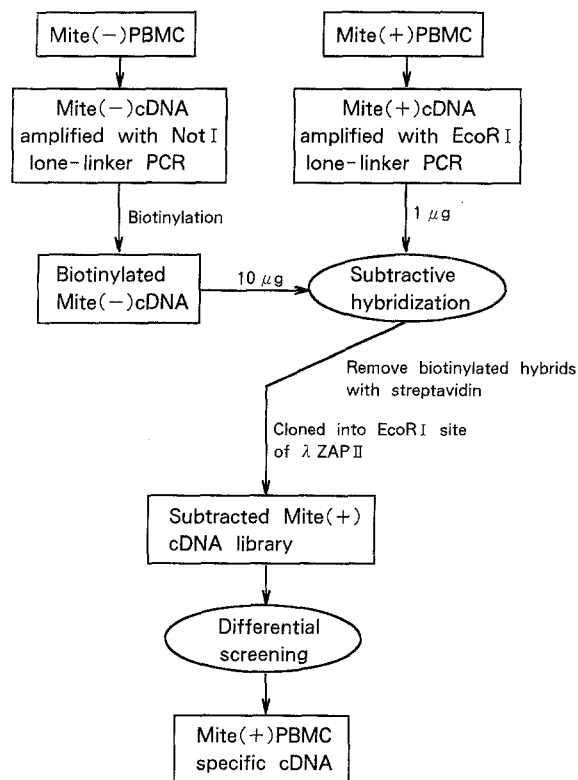


FIG. 2. Subtraction procedure schematic diagram.

formulate the subtracted mite-positive PBMC cDNA library (Fig. 2).

Differential screening

The subtracted mite-positive PBMC cDNA library was transfected into *Escherichia coli* XL1-Blue to create 2000 plaques. The plaques were blotted onto two nylon membranes (Schleicher & Schuell, Inc., Keene, N.H.), then underwent plaque hybridization, with one membrane having the total mite-positive cDNA library as the probe, and the other, the total mite-negative cDNA library as the probe (Fig. 3). α -Phosphorous 32-labeled deoxycytidine triphosphate was integrated into 1 µg of each of the total cDNA libraries with the random primed DNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany), then underwent radioactive labeling. Plaque hybridization was conducted in accordance with the method described in *Molecular Cloning*.¹³ After plaque hybridization, positive plaques were detected with an imaging analyzer (Fuji Film Co., Tokyo, Japan). The authors picked up plaques that released strong signals with total mite-positive cDNA probes but released no, or extremely weak, signals with total mite-negative cDNA probes. Differential screening was conducted the second time for plaques thus obtained to confirm that a strong signal was released only with total mite-positive cDNA probes.

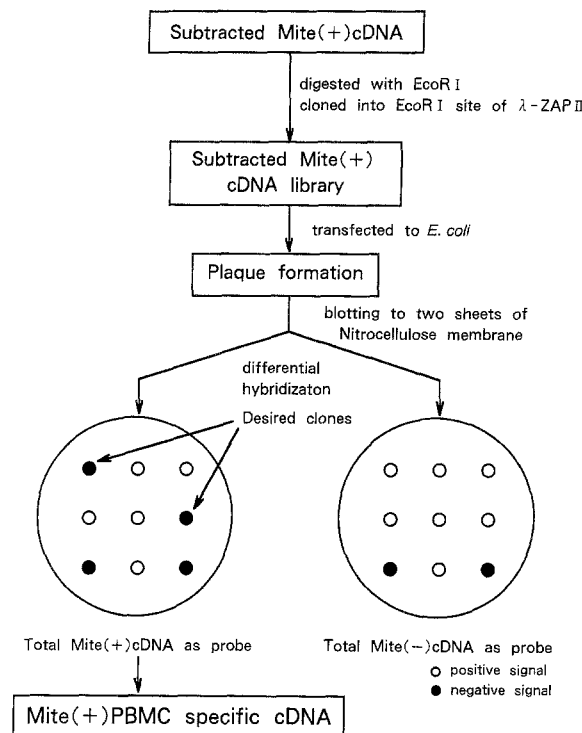


FIG. 3. Outline of protocol for differential screening.

Determination of the cDNA fragment nucleotide sequence inserted into an isolated phage

With an isolated phage as a template, we conducted 25 cycles of PCR, using T3 and T7 located on both sides of λ -ZAPII's cloning sites as the primers, and amplified the inserted DNA fragments. The amplified DNA fragments were purified with a Miniprep Spun Column (Pharmacia), and the nucleotide sequence was determined with the dye terminator method (Applied Biosystem Inc., Chiba, Japan). This nucleotide sequence was searched by means of a computer to check the similarities to and differences from the known genes.

Detection of IL-5 from mite-positive cDNA library

Thirty-five cycles of PCR were performed with approximately 10 ng of the cDNA library formulated with the lone-linker PCR method as templates and 20 bp of each of the two ends of IL-5 as primers. Reactions were as follows: DNA dissociation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and *Taq* DNA polymerase reactions at 72°C for 3 minutes. One tenth of the reaction liquid amount was analyzed by means of agarose gel electrophoresis to check the existence of the band of the expected value. The band that agreed with the expected value of 400 bp was digested with restriction enzyme *Hae*III (Toyobo Co. Ltd., Osaka, Japan) and was confirmed to be broken up into 100 bp and 300 bp.

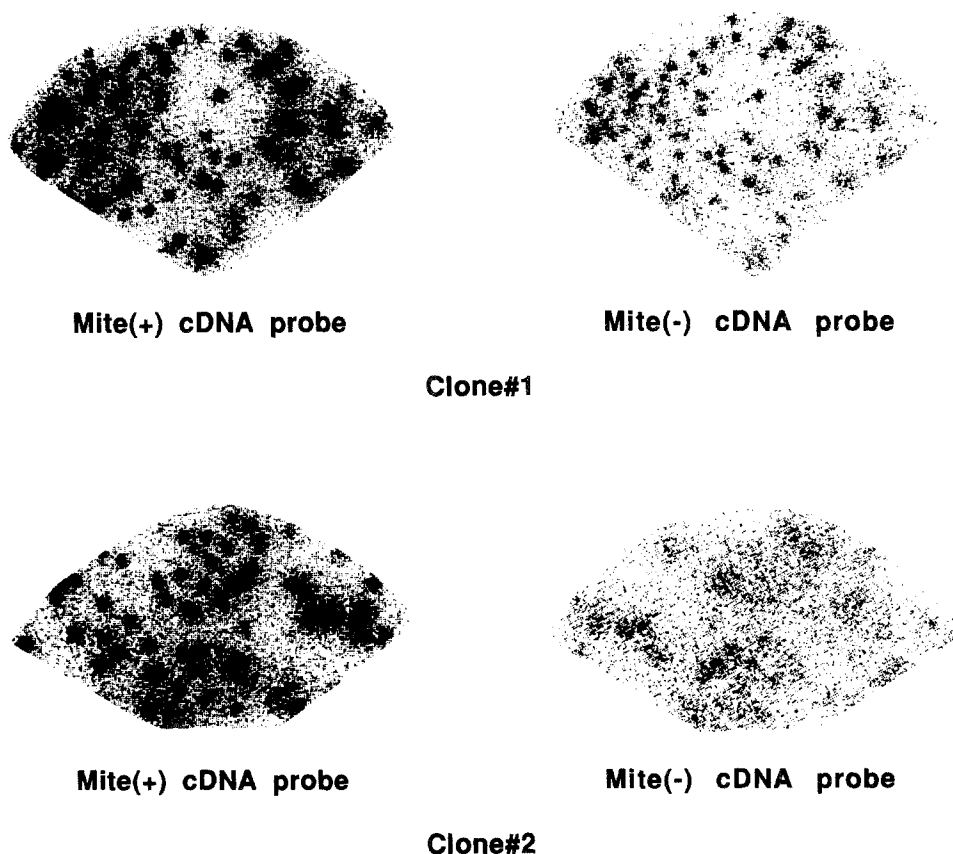


FIG. 4. Confirmation of mite-positive PBMCs specific cDNA with differential hybridization technique. The ^{32}P -labeled total mite-positive cDNAs (*left*) or the ^{32}P -labeled total mite-negative cDNAs (*right*) were hybridized with the mite-positive specific cDNA, which was blotted onto a nylon membrane.

RESULTS

Genes obtained through subtraction and differential screening processes

Two thousand plaques of the cDNA library, in which subtracted cDNA was integrated into λ -ZAPII, were plated and underwent differential screening. As a result, 13 clones were found to possess genes that were strongly expressed in the PBMCs receiving antigen stimulation. Fig. 4 shows the results of the second differential screening, clearly indicating that the signal at the mite-positive cDNA probe is stronger than that at the mite-negative probe. We performed PCR on these 13 clones, amplified the inserted DNA fragments, purified them, and determined the nucleotide sequence.

Determination of the nucleotide sequence and computer search of 13 genes strongly expressed in antigen-stimulated PBMCs

The determined nucleotide sequence was searched by means of a computer, and its similarities to and differences from known genes were

checked. The findings are shown in Table I. Of the 13 genes examined, seven were human mitochondrial genes, and with the exception of ribosomal protein L27, all were genes coded with proteins related to respiratory metabolism. Meanwhile, clones 2 and 4 did not show any homology with known genes and were thus considered unknown genes. Because they showed high homology in their nucleotide sequences, these two genes were considered identical. Both clones contained a polyA tail and polyA-added signal, and approximately 150 bp was obtained upstream from the polyA-added signal. Ordinarily, the distance between the polyA-added signal and termination codon is 100 to 150 bp. Therefore the cDNA obtained here is thought to contain noncoding regions. In addition, the genes for human factor XIII and ferritin were obtained.

Cloning of unknown genes

We decided to clone the coding region of the unknown gene or genes, which were obtained

TABLE I. Sequence homology of subtracted mite-positive cDNA with known genes

Clone no.	Size (bp)	Best score	Homology
1	600	h-Cytochrome oxidase III	96.9% identity in 357nt overlap
2	300	Unknown	
3	650	Rat ribosomal protein L27	90.1% identity in 323nt overlap
4	500	Unknown	
5	500	h-Cytochrome oxidase II	99.3% identity in 281nt overlap
6	550	h-Satellite DNA III	77.4% identity in 266nt overlap
7	500	h-Satellite DNA III	89.7% identity in 156nt overlap
8	400	h-Cytochrome oxidase II	94.0% identity in 334nt overlap
9	600	h-Factor XIII	94.0% identity in 167nt overlap
10	600	h-Ferritin heavy subunit	90.8% identity in 512nt overlap
11	700	h-NADH dehydrogenase	92.7% identity in 396nt overlap
12	1100	h-Cytochrome oxidase II	95.3% identity in 407nt overlap
13	800	h-Cytochrome oxidase II	93.4% identity in 395nt overlap

nt, Nucleotides; *NADH*, reduced nicotinamide adenine dinucleotide.

through differential screening. Before cloning, the existence of the unknown gene or genes in the human placenta cDNA library was examined and confirmed by PCR. Thus we decided to clone the unknown gene or genes contained in the placenta cDNA library. The reason for this was that because the size of the inserted DNA fragment in the placenta cDNA library was bigger than that of the mite-positive cDNA library, it was possible for the former to provide longer strands of the unknown gene cDNA. Plaque hybridization was performed on the human placenta cDNA library, with the already obtained portion of the unknown gene cDNA as the probe. Screening was first done on 180,000 plaques, of which 130 plaques proved positive. Of the positive plaques, 18 with strong signals were selected, and the inserted DNA fragment was amplified through PCR. Finally, the nucleotide sequence for the longest inserted DNA fragment (2 kb) was determined.

Unknown gene nucleotide sequence

The DNA fragments of unknown gene were amplified with PCR, purified with ethanol precipitation, and their nucleotide sequence was determined. As a result of computerized searching, 1200 bp from the tip of this gene indicated close to 100% homology with the region coded with mitochondrial transfer RNA, whereas the 800 bp in the latter half showed no homology with the known genes.

Increase in expression of IL-5 genes in mite-stimulated PBMCs

IL-5 promotes not only the terminal differentiation of the eosinophil from its precursor¹⁴ but also

its migration into the mucosa,¹⁵ its activation¹⁶ and secretion of eosinophil products,¹⁷ and its survival in the tissue.¹⁸ More direct evidence for the role of IL-5 in eosinophilic inflammation in human asthma has been provided by in situ hybridization studies.^{19,20} Therefore the existence of IL-5 cDNA within the mite-positive cDNA library and the mite-negative cDNA library was examined with PCR. As shown in Fig. 5, IL-5 cDNA in the mite-positive cDNA library was amplified, but not that in the mite-negative cDNA library.

Amount of mRNA expression necessary for this cloning method

One clone of a ferritin gene was obtained through subtraction and differential screening. We decided to clarify the amount of ferritin cDNA that exists in the mite-positive cDNA library and the mite-negative cDNA library before subtraction and to determine how much more ferritin cDNA should be expressed by antigen stimulation to enable cloning with the method used herein. After plaque hybridization with ferritin cDNA as the probe was performed, one clone was obtained from 300 plaques of the mite-positive cDNA library, and one clone with a positive signal was obtained from 18,000 plaques of the mite-negative cDNA library. These results suggested that cloning may be possible if the amount of mRNA is increased by 60 times, after antigen stimulation.

DISCUSSION

Thirteen clones were obtained through subtraction and differential screening. Of these, six clones were mitochondrial genes controlling the respira-

tory metabolic system. Probably, this is because the activation of T cells by mite antigen stimulation required an increase in energy production, and thus an increase in the amount of respiratory gene expression occurs. A ferritin gene was also obtained. Ferritin proteins serve as markers for diseases, such as acute leukemia and liver cancer, and the amount of their expression is known to increase when associated with cell dysfunction.^{21,22} Furthermore, it was recently reported that the expression of ferritin mRNA was regulated by a protein factor (iron-responsive element), which in turn is controlled by changes in oxidation/reduction (redox) within the cells.²³ The report indicates that iron-responsive element combines with 5'UTR of ferritin mRNA and controls the translation stage. However, in our study the amount of ferritin mRNA increased 60-fold with mite antigen stimulation, suggesting that in addition to the regulatory functions at the translation stage, iron-responsive element may be involved in the stabilization of mRNA.

The computer analysis showed that cytokine-like genes were not contained in the 13 genes finally obtained in this study. Moreover, in this experiment, even the IL-5 genes could not be isolated by subtraction. As shown in Fig. 5, IL-5 was detected only in mite-positive cDNA, and not in mite-negative cDNA. Thus theoretically, IL-5 should be isolated by subtraction. However, cytokine genes that are expressed in only trace amounts tend to leak out from the net with this method. Because one clone was finally isolated after the ferritin genes were increased by 60 times with mite antigen stimulation, it may be a natural result that the ECA for which activity increased by only 1.8 times with mite antigen stimulation (data not shown) could not be isolated. A great deal of research with the subtraction method has been reported, the majority of which investigates expression changes in genes when either a tissue or an individual body undergoes changes caused by some factors (i.e., induction by developmental stages, infections, and/or stimulation). However, to the best of our knowledge, no report has yet been made on successful isolation of specific target genes by subtraction. It can be concluded from the findings mentioned above that when isolating the target gene by subtraction, between 50 and 100 times more mRNA must be expressed after exposure to stimulants than without exposure to stimulants.

Studies have also been done on other possible approaches available for cloning the ECA genes. Because the protein and DNA structures of ECA are totally unknown, screening cannot be carried

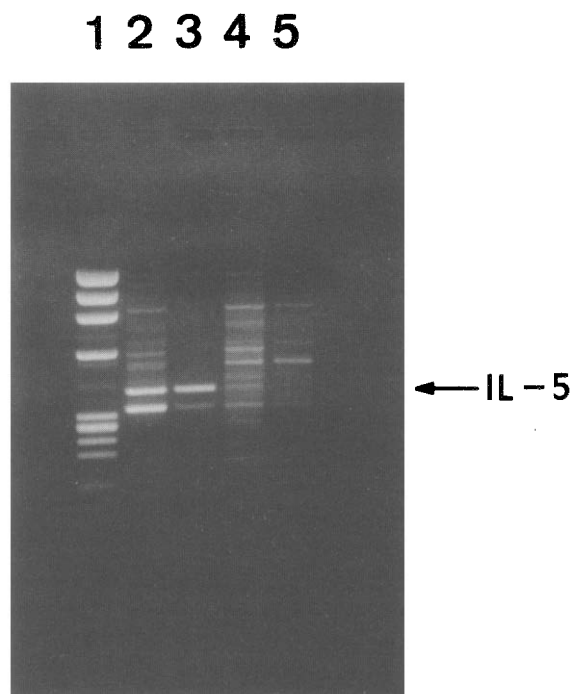


FIG. 5. IL-5 mRNA expressed in mite antigen-stimulated PBMCs. Complementary DNA libraries from mite antigen-stimulated PBMCs and nonstimulated PBMCs were subjected to PCR with IL-5 primers. Lane 1, ϕ x174 *Hae*III fragments; lane 2, mite-positive cDNA with IL-5 primers; lane 3, mite-positive cDNA with IL-5 primers and "perfect match"; lane 4, mite-negative cDNA with IL-5 primers; lane 5, mite-negative cDNA with IL-5 primers and "perfect match." *Perfect match is the name of the reagent that inhibited amplification of nonspecific DNAs in PCRs.

out by plaque hybridization. Moreover, as mentioned in the outline of the experimental method, at least 200 L of blood from asthmatic patients is required to purify the ECA, making this method unfeasible. Regarding expression cloning, meanwhile, it is difficult to obtain sufficient amounts (10^8 order) of PBMCs, which are considered necessary for formulating the cDNA library. In view of this situation, the only remaining method may be to screen the cDNA formulated by using the lone-linker PCR method, with expression cloning with pSP6K vectors. This method combines cDNA with the pSP6K vector's downstream SP-6 promoter; then formulates, approximately, the microgram order of mRNA; translates them inside the oocyte of *Xenopus* species; and checks its activity. This method successfully and effectively clones genes that have been expressed in only trace amounts, such as IL-4²⁴ and IL-5.²⁵ This method is therefore considered to be useful for cloning ECF.

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