

comparative skin testing initially to compare the new standardized extracts to extracts with which he is familiar.

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Connective tissue-activating peptide-III and its derivative, neutrophil-activating peptide-2, release histamine from human basophils

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*Connective tissue-activating peptide-III (CTAP-III) is a 9 kd platelet α -granule-derived growth factor. It stimulates the synthesis of DNA, hyaluronic acid, glycosaminoglycans, and proteoglycan core protein in human fibroblasts. Human mononuclear cell-derived proteases have been previously demonstrated to digest the N-terminal 15 residues of CTAP-III (total, 85 residues) to produce neutrophil-activating peptide-2 (NAP-2). CTAP-III and NAP-2 belong to a class of proteins (platelet factor 4, interleukin-8/NAP-1, etc.) associated with inflammation and wound repair. In our efforts to purify human mononuclear cells and platelet-derived histamine-releasing factors, we had previously discovered that mixtures of CTAP-III and NAP-2 released histamine from human basophils. We have now developed simple protocols for the purification of CTAP-III and NAP-2, independently, from calcium ionophore (A23187)-stimulated platelet supernatants by affinity chromatography and have established their identity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and N-terminal sequence analysis. Each of these released histamine between 2 and 10 $\mu\text{g}/\text{ml}$, a range identical to that obtained with CTAP-III/NAP-2 mixtures that we reported earlier. Thus, our data suggest that CTAP-III and NAP-2 independently release histamine from human basophils in dose ranges similar to ranges required for fibroblast stimulation by each. (*J ALLERGY CLIN IMMUNOL* 1992;89:666-72.)*

Key words: Connective tissue, neutrophil, activating peptides, fibroblast growth factors, histamine release, basophils, protein purification

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HRFs are cell-derived products that interact with basophils or mast cells to cause HR. HRFs were originally described as products of human MNCs.^{1, 2} However, alveolar macrophages,³ platelets,⁴ and neutrophils⁵ were also demonstrated to release HRF. Nasal washings also contain a similar activity.⁶ Most attention has been directed to MNC-derived material (T- and B-lymphocytes and monocytes), and the available evidence suggests that each of these cell types may secrete HRF, although the products of B-lymphocytes may predominate when supernatants from mixed cell populations are studied.⁷⁻⁹ Although human interleukin-3,¹⁰ granulocyte-macrophage colony-stimulating factor,¹⁰ and, to a lesser degree, interleukin-1¹¹ can cause HR from basophils under certain conditions, these appear to be distinct from the major MNC-derived HRFs.^{12, 13}

We have previously reported purification of an HRF from supernatants of stimulated MNCs^{14, 15} that appeared as two closely spaced bands at ≈ 10 kd on SDS-polyacrylamide gels. N-terminal microsequencing of these bands demonstrated that the upper band was identical to CTAP-III and the lower band to NAP-2. CTAP-III is an 85-amino acid polypeptide demonstrated to possess fibroblast growth-factor activity.¹⁶ Found in the α -granules of platelets, it is released when platelets are activated by external stimuli, such as platelet-activating factor, thrombin, calcium ionophore (A23187), and collagen. NAP-2 is a naturally found derivative of CTAP-III, formed by proteolysis of the first 15 (N-terminal) residues,¹⁷⁻¹⁹ and the proteases responsible for this are reported to be derived from both monocytes²⁰ and platelets.¹⁹ In addition, CTAP-III and NAP-2 share a considerable number of structural features with several recently characterized cytokines, such as Chinese hamster embryo fibroblast-GRO, a growth factor constitutively expressed in a Chinese hamster cell line,²¹ g-IP10, a factor induced by interferon- γ in various cell types,²² a factor with melanoma growth stimulatory activity,^{23, 24} IL-8,²⁵ and platelet factor-4.¹⁹ All the above factors have been suggested to play an important role in wound healing and cell growth after tissue injury.²¹

Since our initial HRF preparations contained both CTAP-III and NAP-2, it was unclear whether one or both factors released histamine from basophils. We now report simple protocols for the purification of CTAP-III and NAP-2, independently, from purified platelets and demonstrate that each of these possesses comparable histamine-releasing activity.

METHODS

Platelets and platelet releasate

Platelets were isolated from normal human donors, suspended in PBS containing 1 mmol/L of ethylenediamine-

Abbreviations used

HR:	Histamine release
HRF:	Histamine-releasing factor
MNC:	Mononuclear cell
IL:	Interleukin
CTAP-III:	Connective tissue-activating peptide-III
NAP-2:	Neutrophil-activating peptide-2
MAb:	Monoclonal antibody
PBS:	Phosphate-buffered saline
PMSF:	Phenylmethylsulfonyl fluoride
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MW:	Molecular weight

tetraacetic acid, 5 mmol/L of glucose, and 0.1 mmol/L of PMSF, and stimulated with 20 μ mol/L of calcium ionophore A23187, at 37° C for 30 minutes, according to the method of Collier et al.²⁶ After removal of the cells by centrifugation at 5000 g, the supernatant was dialyzed extensively against PBS, clarified by centrifugation at 15,000 g, and either subjected to affinity chromatography directly or digested with porcine pancreatic elastase (Sigma Chemical Co., St. Louis, Mo.), as described below, and then subjected to affinity chromatography.

Affinity chromatography

Preparation and characterization of MAb, MAb No. 18-1, to CTAP-III and NAP-2 was reported previously.¹⁵ MAb 18-1 was coupled to tressyl-activated agarose (10 mg of IgG per milliliter of Affinica gel, Schleicher & Schuell, Inc., Keene, N.H.) in 0.2 mol/L of NaHCO₃ and 0.5 mol/L of NaCl (pH 8.5) according to manufacturer's recommendations. Chromatography was performed in a siliconized 8 ml glass column (Kontes Glass Co., Vineland, N.J.) at 4° C. The gel was equilibrated with 0.01 mol/L of sodium phosphate buffer, pH 7.5, containing 0.5 mol/L of NaCl, 0.1 mmol/L of PMSF, 0.1 mmol/L of ethylenediaminetetraacetic acid, and 0.02% of azide (equilibration buffer). Platelet releasate was clarified by centrifugation at 15,000 g, followed by addition of PMSF to 0.1 mmol/L. Material equivalent to 15 to 20 mg of protein was applied to the column and washed with the equilibration buffer until the absorbance at 280 nm of the wash fractions returned to the baseline; 0.5 ml fractions were then eluted with 3 mol/L of potassium thiocyanate, and peak protein fractions (monitored by Bradford reagent) were pooled, dialyzed against PBS (pH 7.4) containing 0.1 mmol/L of PMSF, and stored at -70° C in 50 to 100 μ l aliquots.

Electrophoresis, gel staining, and immunoblotting

SDS-polyacrylamide gradient gel electrophoresis was performed according to the method of Laemmli.²⁷ After electrophoresis, protein bands were transblotted onto polyvinylidene difluoride membranes according to the method

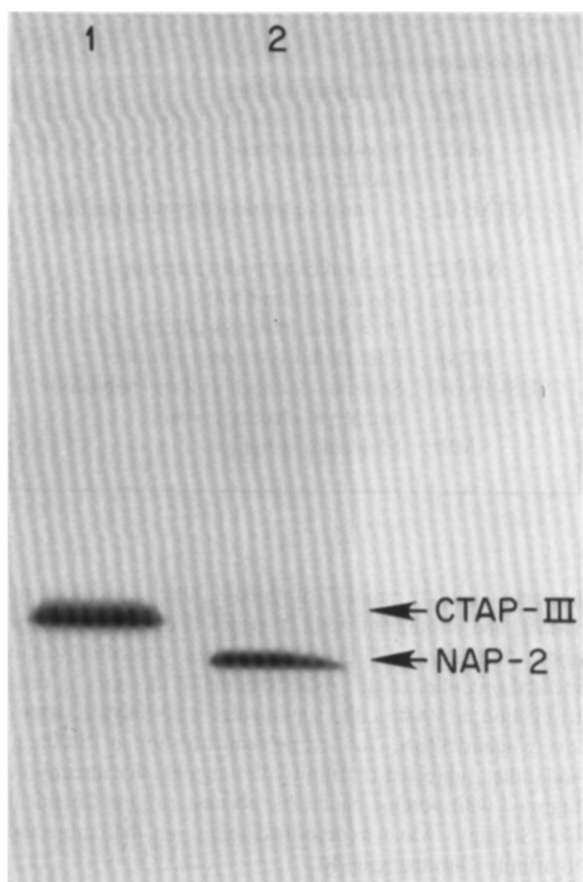


FIG. 1. SDS-PAGE of purified CTAP-III and NAP-2. CTAP-III (lane 1) and NAP-2 (lane 2) were purified as described in METHODS, and 5 μ g of each was electrophoresed under nonreducing conditions on a 10% to 25% gradient polyacrylamide gel and stained with Coomassie brilliant blue R-250.

of Towbin et al.²⁸ Protein bands were stained with amido black, cut out, and subjected to microsequencing according to the method of Matsudaira²⁹ on an Applied BioSystems sequencer (CASM, S.U.N.Y., Stony Brook, N.Y.). Immunostaining was performed as outlined below. After the transfer, unoccupied sites on polyvinylidene difluoride membranes were blocked with BLOTTO³⁰ for 1 hour at room temperature, and treated with 1 μ g/ml of MAb 18-1¹⁵ in BLOTTO for 2 hours. The membranes were then washed four times with BLOTTO during a period of 1 hour, treated with goat antimouse IgG-alkaline phosphatase (Jackson ImmunoResearch Labs, Inc., West Grove, Pa.) at a dilution of 1:1000 in BLOTTO, and washed with BLOTTO four times (15 minutes each wash). Bands were developed with a substrate kit specific for alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

Basophil HR and assay of histamine

Procedures for basophil isolation and HR have been previously described.¹² The radioenzymatic assay for histamine was performed as previously described.³¹

RESULTS

Fresh platelets were obtained from platelet-pheresis packs, as described in METHODS. After purification by differential centrifugation, their purity was checked by light microscopy and was found to be >99%. No MNCs were visible. These platelets were then stimulated with calcium ionophore A23187 to release the contents of their α -granules. The supernatant from this step was dialyzed and subjected to immunoaffinity chromatography with MAb 18-1, and the purity of the bound protein was checked by SDS-PAGE after elution. As illustrated in Fig. 1 (lane 1), the factor appeared essentially homogeneous with an apparent MW of 9 kd, as opposed to the relatively diffuse pattern (because of the presence of both NAP-2 and CTAP-III) previously reported when we purified the factor from stimulated MNCs contaminated with platelets.¹⁵ The identity of the purified species was also checked by N-terminal sequence analysis. The results (not presented) indicated that there was a complete match between the N-terminal sequence of CTAP-III purified by us and the published sequence of CTAP-III.

NAP-2 was purified as follows: The platelet releasate, which usually contained 0.4 to 0.5 mg/ml of protein, was incubated with 0, 0.104, 0.208, 0.415, and 0.83 U/ml of porcine pancreatic elastase at 37° C for 1 hour to determine the optimum enzyme/substrate ratio. At the end of the incubation period, aliquots were immediately added to SDS-PAGE sample buffer, boiled for 5 minutes, and subjected to immunoblotting with MAb 18-1, as described in METHODS to check for conversion of CTAP-III to NAP-2. As illustrated in Fig. 2, A, all CTAP-III was converted to a lower MW form at 0.8 U/ml elastase concentration. Next, we incubated 1 ml of platelet releasate with 0.8 U/ml of elastase at 37° C for 0, 10, 20, 30, 40, 50, 60, and 70 minutes; 20 μ l aliquots were subjected to SDS-PAGE and immunoblotting, as described above. As illustrated in Fig. 2, B, the optimum time for complete conversion of CTAP-III was 60 minutes. It is our observation that different batches of elastase have different specific activities, and therefore, each batch should be evaluated as described above for optimal conditions. We then incubated 15 to 20 ml of platelet releasate with 0.8 U/ml of elastase for 60 minutes at 37° C, and after inactivation of elastase with 1 mmol/L of PMSF, subjected the digest to affinity chromatography on an anti-CTAP-III-Sepharose column, as described in METHODS. The final preparation appeared homogeneous on SDS-polyacrylamide gels (Fig. 1, lane 2) with an MW of 7.5 kd. Its identity with NAP-2 was established by subjecting the preparation to amino acid analysis and N-terminal sequencing (10 cycles)

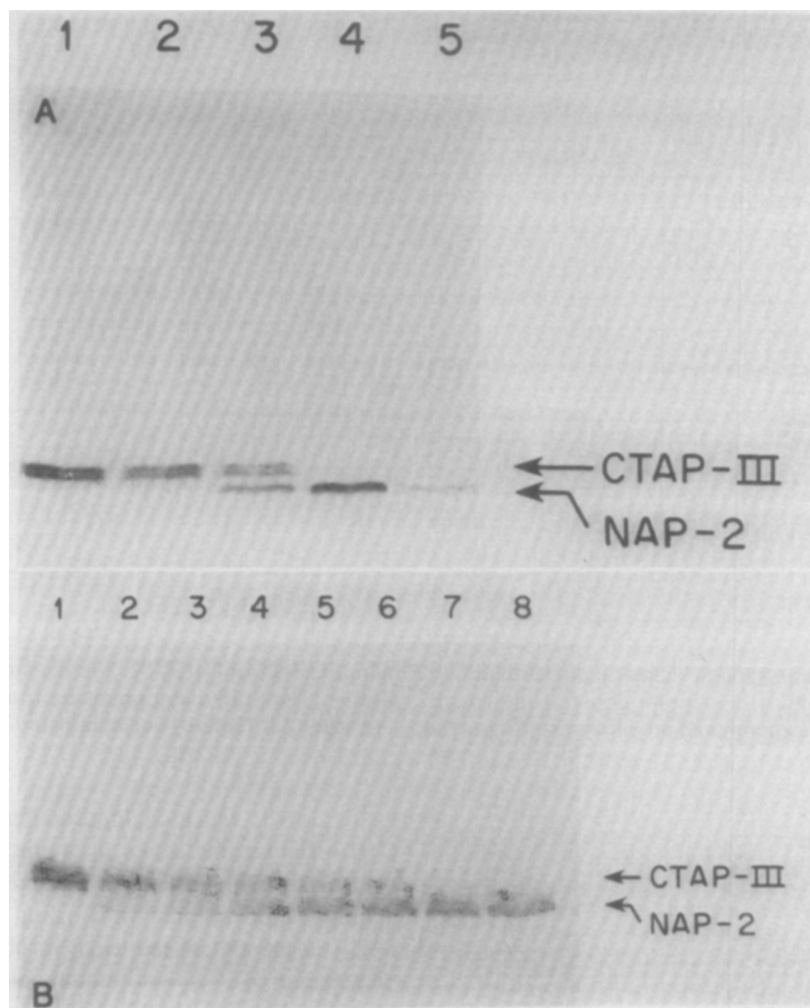


FIG. 2. A, Conversion of CTAP-III to NAP-2 by digestion of crude platelet releasates with porcine pancreatic elastase; 1 ml of calcium ionophore-stimulated platelet releasate, containing 0.5 mg/ml of protein, was incubated with 0 (lane 1), 0.104 (lane 2), 0.208 (lane 3), 0.415 (lane 4), and 0.83 (lane 5) U/ml elastase for 60 minutes at 37° C followed by SDS-PAGE and immunoblotting with anti-CTAP-III MAb. **B,** Time course of digestion with 0.8 U/ml of elastase; lanes 1 to 8: 0, 10, 20, 30, 40, 50, 60, and 70 minutes at 37° C followed by SDS-PAGE and immunoblotting.

as described in METHODS and comparing it to the published sequence of NAP-2. The results (not presented) indicated that the purified NAP-2 had the same amino acid sequence as that published.

These data indicate that porcine pancreatic elastase can be successfully used to purify NAP-2 by specific cleavage of the first 15 residues of CTAP-III. Combined with our MAb 18-1 affinity chromatography, the present protocols yield microgram quantities of pure CTAP-III or NAP-2 in a single step from calcium ionophore-stimulated platelet releasates.

Since the HRF previously reported by us contained both CTAP-III and NAP-2,¹⁵ it was not clear if the two factors elicited HR from the basophils indepen-

dently or in cooperation. When we tested these factors independently in the current study, each released histamine in a dose-dependent fashion. As illustrated in Fig. 3, the HR elicited by the two proteins was parallel, ranging from 4% to 16%. In the same experiment, the recombinant form of NAP-2¹⁹ was also able to release histamine, and its activity was higher than that by either CTAP-III or native NAP-2. Next, we tested 19 different subjects, both atopic and nonatopic for HR with 10 µg/ml of CTAP-III and NAP-2. Data presented in Table I indicate that both factors were capable of releasing histamine from human basophils. The release ranged from 0% to 31.1% (mean ± SEM, 10.1 ± 1.8) for NAP-2, and 2% to

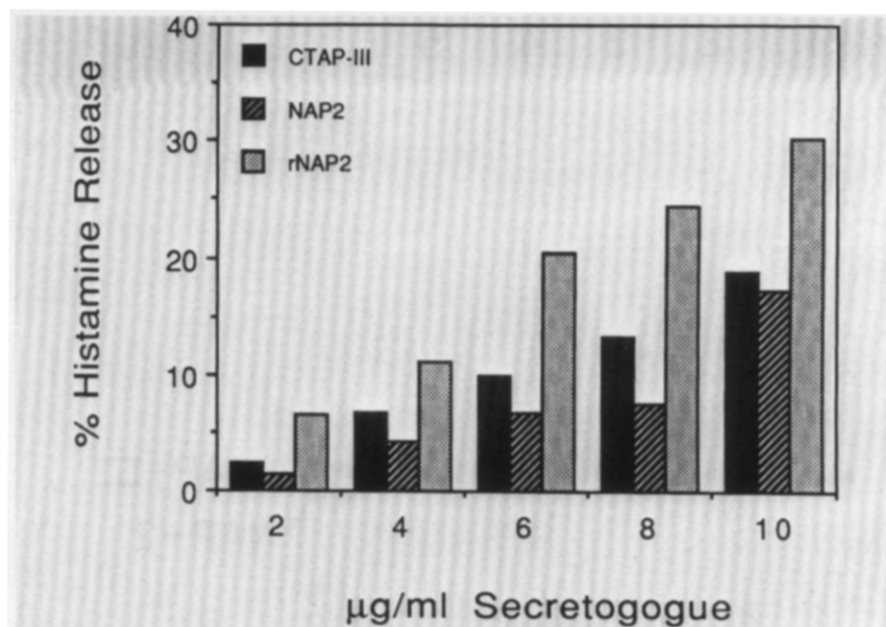


FIG. 3. Representative experiment comparing HR by purified CTAP-III, NAP-2, and recombinant NAP-2 with a responsive donor as described.¹⁴ Mean of duplicate determinations from two separate experiments are illustrated. Spontaneous release of <1% has been subtracted.

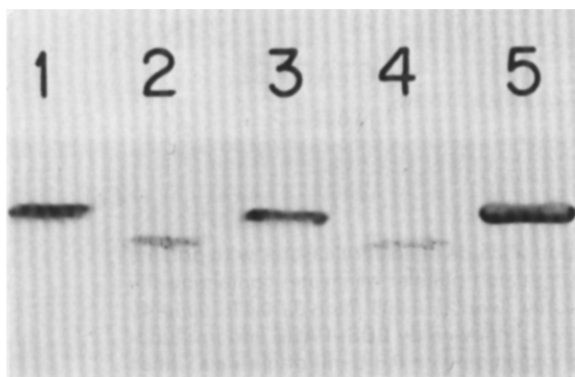


FIG. 4. Stability of CTAP-III added to leukocytes. CTAP-III was incubated with leukocytes to release histamine as described above, and at the end of the incubation period, the reaction mixture was subjected to SDS-PAGE and immunoblotting with MAb 18-1; *lane 1*, CTAP-III alone; *lane 2*, NAP-2 alone; *lane 3*, cells plus CTAP-III; *lane 4*, cells plus NAP-2; and *lane 5*, cells plus CTAP-III (same as *lane 3*).

69% for CTAP-III (mean, 20.1 ± 3.7). In comparison, anti-IgE released 4.4% to 65% histamine with a mean of 38.8 ± 4.7 . These data also suggested that there was no correlation between the allergic status of the basophil donors and HR by CTAP-III and NAP-2.

Next, we wanted to determine whether CTAP-III was being converted to NAP-2 during incubation with

the basophils, which could mean that NAP-2, but not CTAP-III, was the actual histamine releasing agent. We therefore subjected the HR reaction mixtures (containing leukocytes and CTAP-III or NAP-2) to SDS-PAGE at the end of the usual incubation period (45 minutes at 37° C) and immunoblotted with MAb 18-1, as described in METHODS. As illustrated in Fig. 4, CTAP-III and NAP-2 remained indistinguishable from the control incubations (no leukocytes in the incubation mixture, *lanes 1* and *2*), indicating that the interaction of CTAP-III with leukocytes did not result in any detectable leukocyte-mediated conversion of CTAP-III to NAP-2.

DISCUSSION

We have previously demonstrated that mixtures of human MNCs and platelets yield three structurally unrelated histamine releasing activities at MWs 8 to 10 kd, 15 to 17 kd, and 40 kd.¹⁴ Primary structural data are available only for the lowest MW form whose activity is attributable, in part, to CTAP-III and its degradation product, NAP-2.¹⁵ Our present study demonstrates that CTAP-III is the predominant member of this family of proteins in A23187-stimulated platelet releasates (prepared as described in our METHODS), since our MAb did not recognize any band corresponding to the parent molecule platelet basic protein (confirmed by N-terminal sequence analysis). Since platelet releasates prepared under different conditions of intrinsic protease inhibition were reported to contain

only $\approx 13\% \pm 4\%$ platelet basic protein,³² the doublet observed in Fig. 2, A (lane 1) may represent CTAP-III and one of its lower MW N-terminal forms: des 1 to 4 CTAP-III, des 1 to 13 CTAP-III, or des 1 to 14 CTAP-III. These forms have been observed in outdated platelets.^{17,19} However, irrespective of the nature of the intermediate CTAP-III variant observed in Fig. 2, A, the final digestion product yielded a single band and unequivocal N-terminal sequence of NAP-2.

Our data reveal that both these factors are capable of releasing histamine from basophils (Fig. 3 and Table I). The effective range (0.2 to 1 $\mu\text{mol/L}$) was the same as that required for the stimulation of glycosaminoglycan and DNA synthesis in fibroblast cultures.¹⁹ By contrast, NAP-2 and IL-8 (NAP-1) exhibit comparable specific activities on neutrophils, whereas CTAP-III has little or no activity.^{20,33} Since monocyte-derived proteases have previously been demonstrated to convert CTAP-III to NAP-2²⁰ and the leukocytes used in our HR experiments may contain monocytes, NAP-2 might be the histamine releaser in CTAP-III experiments. Data illustrated in Fig. 4 preclude that possibility and suggest that both CTAP-III and NAP-2 can independently release histamine from basophils. Curiously, the recombinant form of NAP-2 was relatively more active than the native form as an HRF, and this finding was reminiscent of the higher neutrophil-activating activity of the recombinant form reported by Castor et al.³⁴ Also, since IL-8 (NAP-1) does not release histamine under identical conditions,³⁵ it is our belief that HR caused by NAP-2 is not due to neutrophil-derived factors.

It is important to note that the present HR experiments did not include deuterium oxide (which non-specifically augments basophil responsiveness two-fold to threefold) used in our earlier studies. Although micromolar quantities of CTAP-III and NAP-2 are apparently required for significant HR, these two platelet α -granular proteins are present at 1000 times the concentrations of other cytokines¹⁹ and can therefore contribute significantly to HR after platelet activation, which has been observed in allergic and asthmatic disease states.³⁶⁻³⁹

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TABLE I. HR (percent) by CTAP-III and NAP-2

Donor	NAP-2	CTAP-III	Anti-IgE
1 J. S.	10.0	24.0	51.5
2 D. L.	10.3	20.1	26.5
3 P. D.	8.8	25.3	53.2
4 P. K.	14.3	17.9	47.1
5 P. M.	11.5	26.0	57.9
6 L. P.*	16.6	4.0	58.5
7 D. R.*	31.1	69.0	55.0
8 G. M.*	13.7	2.0	54.1
9 E. S.	13.5	25.0	30.2
10 G. R.	15.4	15.0	29.9
11 G. G.	13.3	16.2	51.7
12 E. M.*	17.4	17.5	13.2
13 D. A.*	3.6	6.5	65.0
14 D. C.*	0.0	36.0	46.0
15 J. G.*	7.4	15.0	60.6
16 M. M.	1.0	14.0	8.0
17 S. R.*	0.0	41.0	7.2
18 H. L.	3.0	4.6	4.4
19 S. L.	1.7	2.6	17.0

*Atopic basophil donor based on family history and positive skin tests.

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