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Immunology

Hypersensitivity to tobacco antigen

(cigarette smoking/allergy/cardiovascular diseases/skin tests)

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ABSTRACT **A glycoprotein of molecular weight 18,000 was purified from saline extracts of cured tobacco leaves by ammonium sulfate fractionation, chromatography on Sephadex G-25, and continuous-flow preparative electrophoresis on polyacrylamide gel. Twelve of 31 volunteers ($^6/_{15}$ smokers and $^6/_{16}$ nonsmokers) exhibited immediate cutaneous hypersensitivity (wheal and flare reactions) when injected intracutaneously with 2 μg of this material. Immunochemically similar material was demonstrated in, and purified from, cigarette smoke condensate and cigarette smoke. The concentration in cigarette smoke condensate ("tar") was determined to be 1.8–3.6 mg/g. Antigenically crossreactive material was also demonstrated in eggplants, green peppers, potatoes, and tomatoes which, like tobacco, are members of the family *Solanaceae*.**

Immediate cutaneous hypersensitivity to extracts of defatted tobacco leaves has been reported previously in smokers and in nonsmokers. Harkavy has suggested that allergy to constituents of tobacco may underly the relationship between tobacco smoking and coronary artery disease and peripheral vascular disease (1, 2). In this connection, Fontana and colleagues have demonstrated that 28% of smokers with immediate cutaneous hypersensitivity to tobacco extracts had changes in peripheral circulation, as measured by change in skin temperature, when they smoked cigarettes as compared with smokers with negative skin tests of whom only 4% had changes in skin temperatures (3).

However, the concept of allergy to tobacco constituents has not been generally accepted because (*a*) many nonsmokers were also found to be hypersensitive; (*b*) it was not clearly demonstrated that antigens extractable from tobacco leaf were also present in tobacco smoke or, if present, in what quantity; (*c*) antigens from tobacco leaves or from concentrated tobacco smoke were far from pure and may have included noxious material capable of inducing a wheal and flare reaction when injected intracutaneously.

We report here on experiments in which an 18,000 molecular weight glycoprotein was purified from saline extracts of cured tobacco leaves (*N. tobacum*) by $(NH_4)_2SO_4$ fractionation, chromatography on Sephadex G-25, and preparative continuous-flow electrophoresis on alkaline polyacrylamide gel. This material will be referred to as tobacco glycoprotein (TGP). Twelve of 31 human volunteers displayed immediate hypersensitivity reactions when injected intracutaneously with TGP. Similar material was demonstrated in the four commonly used varieties of tobacco leaf. In addition, a similar glycoprotein was purified from cigarette smoke condensate, and from saline through which cigarette smoke had been bubbled. By hemagglutination inhibition, it was demonstrated that TGP and material purified from cigarette smoke condensate and from saline extracts of cigarette smoke were immunochemically similar, if not identical. It was also observed by hemagglutination inhibition that other members of the family *Solanaceae* besides tobacco, including eggplant (*S. melongena*), green pepper (*C. frutescens*), potato (*S. tuberosum*), and tomato (*L. esculentum*), contain crossreactive antigen with electrophoretic mobility, in alkaline polyacrylamide gels, similar to tobacco glycoprotein.

Abbreviation: TGP, tobacco glycoprotein.

MATERIALS AND METHODS

Tobacco Leaves. Cured tobacco leaves of Virginia Bright, Burley, Maryland, and Turkish (*Xanthis*) varieties were supplied by the American Tobacco Co. Virginia Bright leaves, divided into strip and stem portions, were also supplied by the Phillip Morris Tobacco Co.

Purification of Tobacco Glycoprotein Antigen (TGP). (*i*) Virginia Bright strips (100 g) were powdered in a Waring blender and defatted by stirring for 24 hr with petroleum ether. The leaves were air-dried after removal of petroleum ether and extracted for 24 hr with 1 liter of phosphate-buffered physiological saline at pH 7.4, which contained 0.4% phenol as preservative. Solid material was removed by filtration through Whatman no. 3 filter paper and the solution further clarified by centrifugation at 43,000 × *g* in a Sorvall RC-2B centrifuge. (*ii*) The clarified solution was concentrated to 200 ml by pressure dialysis through Amicon PM-10 membranes (Amicon Corp., Lexington, Mass.) and brought to 50% $(NH_4)_2SO_4$ saturation. The brown precipitate was collected by centrifugation, redissolved in, and dialyzed against distilled water, and lyophilized. (*iii*) Approximately 50 mg of the lyophilized powder was then redissolved in 5 ml of phosphate-buffered saline and applied to a fine Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column measuring 2.5 × 100 cm, equilibrated with phosphate-buffered saline containing 0.1% NaN_3. Brown material emerging with the void volume was collected, concentrated by pressure dialysis and rechromatographed. On the second passage a single symmetrical peak as measured by absorbance at 280 nm emerged with the void volume with little or no trailing. This peak, containing brown material, was dialyzed against distilled water and lyophilized. (*iv*) The lyophilized powder (30–40 mg) was dissolved in 3 ml of Tris·HCl buffer at pH 8.9 which was made 20% in sucrose, and applied to the top of a polyacrylamide gel column consisting of a 7.5% separating gel and a 3.5% stacking gel. The buffers in upper and lower chambers, and in the separating and stacking gels, were identical to those described by Davis and Ornstein (4). The dimensions of the separating gel and stacking gel were 8 × 2.5 cm and 2.4 × 2.5 cm, respectively.

At the bottom of the gel was a polypropylene filter beneath which was a Teflon elution capsule measuring 2.5 × 0.2 cm which was separated from the lower buffer by Visking dialysis membrane. Lower buffer was drawn with a peristaltic pump from a separate container through the elution capsule, and routed through an LKB ultraviolet scanner (280 nm) into a fraction collector. Volumes of 5 ml per tube were collected. Electrophoresis was carried out at 250 V and 50 mA at 20°. The

TGP migrated rapidly as a discrete brown band through both stacking and separating gels and was collected in approximately two to three tubes after being recorded as a sharp symmetrical peak. Its electrophoretic mobility was between that of bromphenol blue and of serum albumin. A second brown band was trapped at the top of the separating gel, and an opaque band was trapped at the top of the stacking gel. The fraction which emerged from the gel was dialyzed exhaustively against distilled water and lyophilized. The markedly hygroscopic powder was stored at room temperature.

Extraction of Varieties of Tobacco and of Vegetables. (*i*) Tobacco leaves of Burley, Maryland, and Turkish (*Xanthis*) varieties were powdered, defatted and extracted with phosphate-buffered saline as described above. The brown precipitate formed at 50% $(NH_4)_2SO_4$ fractionation was dialyzed exhaustively against distilled water and lyophilized. These were not subjected to further purification. (*ii*) Whole eggplants, green peppers, potatoes, and tomatoes were ground in a Waring blender in phosphate-buffered saline containing 0.4% phenol. The resulting brie was stirred in phosphate-buffered saline for 24 hr. The solution was clarified by filtration through Whatman no. 3 filter paper and centrifugation at 43,000 × *g*. The clarified solutions were extracted twice with equal volumes of petroleum ether in a separatory funnel. The saline infranatants were collected and placed in a water bath at 56° to volatilize any residual petroleum ether, and filtered once more through Whatman no. 3 filter paper. The solutions were then brought to 50% $(NH_4)_2SO_4$ saturation. The precipitates were collected by centrifugation, dissolved in distilled water, dialyzed exhaustively against distilled water, and lyophilized.

Polyacrylamide Gel Electrophoresis. This was performed on disc polyacrylamide gel apparatus (Buchler Instruments Co., Fort Lee, N.J.) using a 7.5% acrylamide separating gel, a 3% stacking gel and a discontinuous Tris·HCl buffer system at pH 9.3 as described by Davis and Ornstein (4). The same gel concentrations and buffer system were also used later in slab polyacrylamide gels using a Hoefer slab gel apparatus (Hoefer Instruments Co., San Francisco, Calif.). Gels were stained with Coomassie brilliant blue or by the periodic acid-Schiff technique.

Molecular Weight Determinations. Molecular weight determinations of TGP and of material isolated from cigarette smoke condensates and cigarette smoke saline were performed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels as described by Weber and Osborn (5). Substances of known molecular weight were obtained from Schwarz Mann Co., Orangeburg, N.Y., and included horse heart cytochrome *c* (12,400), sperm whale myoglobin (17,800), beef pancreas chymotrypsinogen (25,000), ovalbumin (45,000), and bovine albumin (67,000). Molecular weight was also determined by electrophoresis in alkaline polyacrylamide slab gels of 7.5, 10, 12.5, and 15% polyacrylamide concentration. The proportion of bis-acrylamide to acrylamide was constant. The buffers used were as described above. Purified TGP and cigarette smoke extracts and molecular weight markers were electrophoresed at 100 mA for 3 hr. The gels were fixed in 12.5% trichloroacetic acid, washed, and stained with Coomassie brilliant blue. The distances each substance migrated from the origin were measured. The slope of distance migrated versus polyacrylamide gel concentration was calculated for each substance using mean of least squares technique. These slope values were then plotted against molecular weight and a second slope value calculated.

Amino Acid Analysis. Amino acid analysis of TGP purified from flue cured Virginia Bright leaves was performed by Worthington Biochemical Corporation, Freehold, N.J.

Iron Content of TGP. The iron content of purified TGP was determined by the method of Peters *et al.* (6).

Cigarette Smoke Condensate and Cigarette Smoke Saline. Cigarette smoke condensate, produced from University of Kentucky 1R1 cigarettes smoked in a standard manner, was obtained through the courtesy of Dr. M. R. Guerin of Oak Ridge National Laboratories, Oak Ridge, Tenn. (7, 8). Saline extracts of cigarette smoke were made by attaching cigarettes to a manifold and bubbling cigarette smoke through a vacuum flask containing phosphate-buffered saline.

Cigarette smoke condensate (9 g) was shaken for 24 hr with 15 ml of phosphate-buffered saline which contained 1% Tween 20. The suspension was clarified by filtration and centrifugation as described above, and extracted three times with 100 ml volumes of petroleum ether in a separatory funnel. The saline infranatant was placed in a water bath at 56° for 1 hr to volatalize any residual petroleum ether. A portion of this material was saved and will be referred to as crude cigarette smoke condensate extract. Saline extracts of cigarette smoke were concentrated by pressure dialysis on an Amicon P-10 filter and processed similarly. The remainder was subjected to ammonium sulfate fractionation, chromatography on Sephadex G-25, and preparative electrophoresis in polyacrylamide gel dialysis and lyophilization, as described above. Material purified this way will be referred to as cigarette smoke condensate antigen or pure cigarette smoke saline antigen.

Quantitation of TGP. The quantity of pure TGP in solution was measured by the Lowry modification of the method of Folin and Ciocalteau (9). A standard curve was constructed using purified TGP in which the values obtained by the Lowry procedure were compared with protein nitrogen determinations performed according to the Markham modification of the micro Kjeldahl technique (10).

Immunization of Rabbits. Young adult New Zealand red and Chinchilla rabbits were each injected intracutaneously at two sites on their backs with 1 mg of pure TGP in complete Freund's adjuvant. One and two weeks later they were injected intravenously with 1 mg of purified TGP precipitated with alum. Even after several courses of immunization over several months, it was not possible to demonstrate precipitin reactions between TGP and the rabbit antiserum. Antibody to TGP was measured by hemagglutination using tanned human type O erythrocytes coated with TGP.

Hemagglutination and Hemaglutination Inhibition Assays. Washed human erythrocytes of blood group O were tanned and coated with pure TGP according to methods described by Herbert (11). Rabbit antisera to TGP and pooled normal rabbit serum were heat inactivated and absorbed with washed, packed, natural and tanned type O erythrocytes to remove any hemagglutinins active against antigens on untreated O cells or neoantigens which might have been exposed by tanning. Serum dilutions were prepared in Microtiter U-plates, (Cooke Laboratory Products, Alexandria, Va.). The titre of anti-TGP antibodies ranged between 1:256 and 1:1024.

Hemagglutination inhibition assays were performed according to methods described by Kwapinski (12). Because of the possibility that some plant extracts also contain lectins which might react with either erythrocytes or plasma proteins and produce false-positive hemagglutination inhibition reactions, tanned human type O erythrocytes were coated with human gamma globulin and a hemagglutination system was constructed using heavy chain specific rabbit antisera to human IgG. Thus, TGP, other tobacco extracts, extracts of other members of the family *Solanaceae*, and material purified from cigarette smoke condensate and cigarette smoke saline were

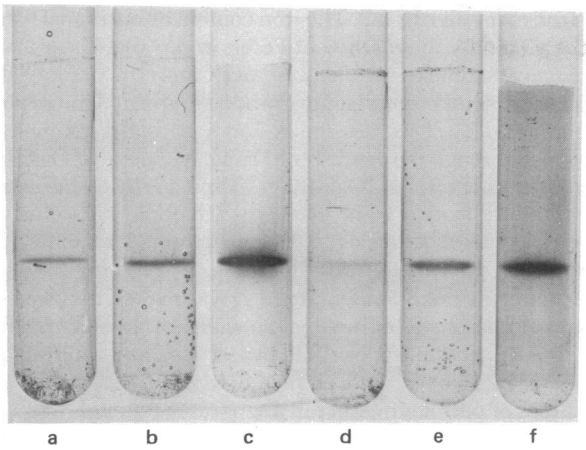

FIG. 1. Electrophoresis in alkaline 7.5% polyacrylamide gels of purified TGP (gels a–c) and of material purified from cigarette smoke condensate (gels d–f). Gels (a) and (d) are unstained and the bands are brown; gels (b) and (e) are stained with Coomassie brilliant blue; and gels (c) and (f) are stained by the periodic acid-Schiff reaction. It can be seen that TGP and material purified from cigarette smoke condensate are electrophoretically and tinctorially identical.

measured (*a*) for their capacity to inhibit agglutination of TGP coated erythrocytes by rabbit antisera to TGP and (*b*) for any nonspecific inhibition of agglutination of human gamma globulin coated erythrocytes by rabbit antisera to human IgG heavy chains.

Intracutaneous Injection of Human Volunteers with Purified TGP. Human volunteers, to whom the procedure had been explained and who gave consent, were injected intracutaneously on the external surface of the upper arm with 2 μg of purified TGP in 0.05 ml of sterile saline. Sterile saline was injected in a second site several centimeters removed as control. The inoculation sites were observed for 30 min for the development of wheal and flare reactions. These generally developed within 5–15 min. The diameters of both wheal and flare were measured when such responses occurred.

The volunteers included both smokers and nonsmokers. The former each smoked approximately one pack of cigarettes per day.

RESULTS

Electrophoretic and Molecular Weight Studies. Purified TGP migrated as a single band when 50 μg was applied to alkaline polyacrylamide gels. The material was brown in unstained gels and could be stained with Coomassie brilliant blue and the periodic acid-Schiff technique. No additional bands were revealed by these stains (Fig. 1). Identical electrophoretograms were obtained with material extracted from Burley, Maryland, or Turkish varieties of tobacco. Brown material electrophoretically and tinctorially similar to TGP was demonstrated in saline extracts of cigarette smoke condensate and in cigarette smoke saline which had been subjected to ammonium sulfate fractionation and chromatography on Sephadex G-25. This anodic migrating material was further purified from cigarette smoke condensate and from cigarette smoke saline by preparative continuous flow electrophoresis on polyacrylamide gels. The molecular weight of pure TGP and of material purified from cigarette smoke condensate by electrophoresis in alkaline acrylamide gels was 18,000. By the sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique the molecular weight was also found to be approximately the same.

Electrophoretically and tinctorially similar bands were observed when extracts of eggplant, green pepper, potato, and tomato were compared with purified TGP on alkaline slab polyacrylamide gels (Fig. 2).

Table 1. Amino acid analysis of purified TGP

Amino acid	Residues per 100 residues
Lysine	5.8
Histidine	2.3
Arginine	2.5
Aspartic acid	9.8
Threonine	4.8
Serine	8.6
Glutamic acid	10.7
Proline	7.3
Glycine	12.0
Alanine	6.9
Half-cystine	6.4
Valine	4.9
Methionine	2.2
Isoleucine	4.6
Leucine	4.9
Tyrosine	3.1
Phenylalanine	2.9

Amino Acid Analysis. Results of amino acid composition of purified TGP are shown in Table 1.

Iron Content. The iron content of TGP was found to be 0.3%.

Hemagglutination Inhibition Assays. It was observed that purified TGP and material similar to TGP purified from cigarette smoke condensate and from cigarette smoke saline were capable of inhibiting agglutination of human type O erythrocytes by rabbit antiserum to TGP (Table 2). The minimum concentration of antigen necessary to inhibit agglutination was essentially the same for TGP (1.91 μg/ml) and for antigen purified from cigarette smoke condensate (1.81 μg/ml) or cigarette smoke saline (1.80 μg/ml) indicating similar if not identical antigenicity. When the amount of antigenic material in saline extracts of crude cigarette smoke condensate was calculated from these and similar experiments in terms of purified TGP, it corresponded to a concentration of between 1.8 and 3.6 mg of antigen per g of cigarette smoke condensate.

Extracts of other varieties of tobacco [Burley, Maryland, and Turkish (*Xanthis*)] similarly inhibited agglutination of TGP coated erythrocytes by rabbit anti-TGP serum.

It was observed that material precipitated at 50% ammonium sulfate fractionation from saline extracts of eggplant, green pepper, potato, and tomato were also capable of inhibiting agglutination of TGP-coated erythrocytes by rabbit antiserum to TGP (Table 3). This result is in harmony with the results of electrophoretic analyses described above. It appeared that extract of potato also contained a hemagglutinin demonstrable

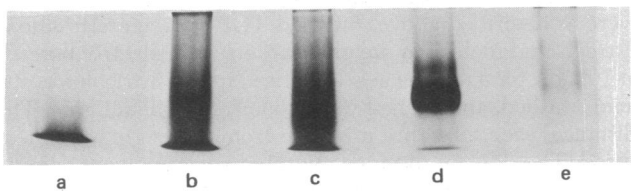

FIG. 2. Electrophoretograms on an alkaline 7.5% polyacrylamide slab gel of purified TGP (a), and delipidized $(NH_4)_2SO_4$ fractionated (50% saturation) extracts of eggplant (b), green pepper (c), potato (d), and tomato (e). The most anodically migrating band of each is brown or tan in unstained preparations and stains with Coomassie brilliant blue and with periodic acid-Schiff reactions.

Table 2. Hemagglutination inhibition assay comparing pure TGP and similar material from cigarette smoke condensate (CSC) and from cigarette smoke saline extracts (CSS)

Antigen	Serum*	Cells	Agglutination	Minimum inhibiting antigen concentration (μg/ml)
TGP	Rabbit anti-TGP	TGP-labeled	–	1.91
CSC	Rabbit anti-TGP	TGP-labeled	–	1.81
CSS	Rabbit anti-TGP	TGP-labeled	–	1.80
PBS†	Rabbit anti-TGP	TGP-labeled	+	
PBS	Rabbit anti-TGP	Control	–	
PBS	Normal rabbit serum	TGP-labeled	–	
PBS	Normal rabbit serum	Control	–	

* Serum was diluted 1:256.
† PBS, phosphate-buffered saline.

in lower dilutions. It is known that potato contains a hemagglutinin (13).

Intracutaneous Inoculation in Human Volunteers. Wheal and flare reactions occurring within 20 min of intracutaneous inoculation of 2 μg of purified TGP were observed in 12 of 31 human volunteers. The greatest diameter of the wheals and of the flares was measured. The mean wheal diameter was 13 mm (range 10–17 mm) and the mean flare diameter was 38 mm (range 25–60 mm). There was no reaction in either reactive or unreactive subjects to the sterile saline control (Fig. 3). The distribution of positive reactions among smokers and nonsmokers is illustrated in Table 4.

DISCUSSION

These results indicate that approximately one-third of human volunteers exhibit cutaneous hypersensitivity to a glycoprotein purified from cured tobacco leaves (TGP) and also present in

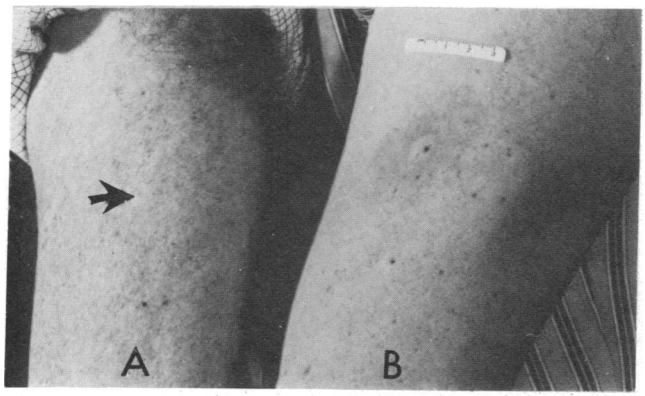

FIG. 3. Demonstration of immediate hypersensitivity to purified TGP. Subject A was inoculated with 2 μg of purified TGP in 0.05 ml of sterile saline (arrow). Sterile saline (0.05 ml) was injected 4 cm below as control. Subject A was unreactive. Subject B was injected similarly. A wheal and flare reaction can be seen at the site of injection of TGP. Photograph was taken 15 min after injection of both subjects.

Table 3. Hemagglutination inhibition assay comparing pure TGP and extracts of eggplant (EP), green pepper (GP), potato (P), and tomato (T)

Antigen	Serum*	Cells	Agglutination	Minimum inhibiting antigen concentration (μg/ml)
TGP	Rabbit anti-TGP	TGP-labeled	–	2.29
EP	Rabbit anti-TGP	TGP-labeled	–	3.00
GP	Rabbit anti-TGP	TGP-labeled	–	7.89
P	Rabbit anti-TGP	TGP-labeled	–	3.66
T	Rabbit anti-TGP	TGP-labeled	–	10.00
PBS†	Rabbit anti-TGP	TGP-labeled	+	
PBS	Rabbit anti-TGP	Control	–	
PBS	Normal rabbit serum	TGP-labeled	–	
PBS	Normal rabbit serum	Control	–	

* Serum was diluted 1:64.
† PBS, phosphate-buffered saline.

cigarette smoke and cigarette smoke condensate. All these sources yielded a brown material with a molecular weight of 18,000 and identical electrophoretic mobilities in alkaline polyacrylamide gels. This material stained with Coomassie blue and periodic acid-Schiff stains, and was cross reactive in hemagglutination inhibition assays using rabbit antibody to TGP. The high incidence of hypersensitivity in both smokers and nonsmokers is comparable to that reported in studies employing tobacco leaf antigen of lesser purity (1–3).

The color, molecular weight, amino acid analysis, iron content, and presence of carbohydrate moiety in TGP all correspond to that of an iron–protein–chlorogenic acid-rutin complex in cured tobacco leaves, first reported by Wright *et al.* (14). This material is presumably the source of the protein complex demonstrated by Stedman *et al.* in cigarette smoke condensate (15, 16).

From the concentration of antigen found in cigarette smoke condensate (1.8–3.6 μg/g) it follows that 20 cigarettes, with 20 mg of available cigarette smoke condensate ("tar") per cigarette, contain between 720 and 1440 μg of antigen, clearly a quantity capable of sensitizing the smoker, and perhaps adjacent nonsmokers.

In addition to tobacco, other members of the family *Solanacea* which we have tested (eggplant, green pepper, potato, and tomato) contain a brown material electrophoretically similar to TGP and cross reactive with rabbit antibodies to TGP.

Table 4. Immediate cutaneous hypersensitivity to TGP

	Smokers	Nonsmokers	Total
Skin test positive	6	6	12
Skin test negative	9	10	19
			31

This suggests that hypersensitivity to TGP may also result from ingestion of cross reactive antigens in commonly consumed vegetables. Thus, the high incidence of cutaneous hyperreactivity to tobacco leaf extracts and to purified TGP may well be a function of antigenic ubiquity (rather than nonspecificity), since the antigen is in smoke-contaminated air and frequently eaten foods.

Data presented here support, in several ways, the hypothesis that allergy to tobacco constituents may be the basis of the association between cigarette smoking and vascular disease: (*a*) approximately one-third of people tested are shown to be hypersensitive to a highly purified antigen present in tobacco; (*b*) significant quantities of the antigen are in cigarette smoke, each pack exposing the smoker to about 1 mg of antigen; (*c*) when inhaled into the lungs, the relatively small size of the antigen (18,000 molecular weight) may allow easy access to the circulating blood.

It is conceivable that circulating tobacco antigen might, in sensitive subjects, react with antibody to produce focal injury of blood vessels. It is known that the reaction of basophile-bound IgE with specific antigen results in the release of vasoactive amines and of platelet activating factor (PAF). The latter induces aggregation of platelets and the release of vasoactive substances from them (17). It can be speculated that repetitive antigenic challenges by tobacco antigen might, as a consequence of immunologically mediated injury to endothelium or immunologically mediated stimulation of endothelial contraction (18), result in increased permeability of blood vessel endothelium to macromolecules such as lipoprotein. In this connection, it has been shown in this laboratory that repeated injection of foreign protein, and modest hyperlipidemia, act synergistically to induce athero–arteriosclerosis (19).

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