

can also inhibit the synthesis of IgG antibody in an antigen-specific fashion. The mechanism of action of these cells is unknown. They may simply serve to focus antigen on the surface of B cells, which then become inhibited. Alternatively, they may function in concert with additional regulatory subsets to produce specific suppression, as has been shown in antigen-induced IgM antibody synthesis (20, 21). Nevertheless, it would appear that the antigen-induced inhibition of IgG antibody synthesis is modulated by at least 2 lymphocyte subsets. At this time, it is difficult to predict the relationship of these cells with one another and with the *in vivo* immune response.

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ALLERGENICALLY ACTIVE COMPONENTS OF CAT ALLERGEN EXTRACTS

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The allergens involved in cat allergy have been studied in pelt extracts, saliva, serum, and urine. Using crossed immunoelectrophoresis (CIE) to examine the antigenic content, and crossed radioimmunoelectrophoresis (CRIE) and RAST to examine the allergenic content, it was found that allergen 1 of Dr. Ohman is the most important allergenic component, whereas albumin and several unidentified proteins play a minor role. Allergen 1 was not detectable in serum and urine. The allergenic and non-allergenic proteins of pelt extract and saliva were identi-

cal by CIE, suggesting that pelt extract proteins are mainly of salivary origin.

Several years ago we published a preliminary report of our studies on cat allergens (1), in which it was demonstrated that cat pelt, saliva, serum, and urine exhibited allergenic activity. The allergenic and antigenic components of each of these sources of allergens were not studied nor was their relative importance for cat allergy evaluated. The present study indicates the presence of at least 8 antigens in cat hair-dander-pelt extracts and cat saliva. Of the human sera tested, specific IgE antibody was in highest titer to allergen 1 (2), but there was also specific IgE antibody to albumin and to at least 2 other proteins.

MATERIALS AND METHODS

Two pelt extracts, Lots 206 and 307, were prepared by Center Laboratories, Inc., in 1975 as a contribution to the program on standardization of

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allergens of the subcommittee on allergen standardization of the International Union of Immunological Societies (I. U. I. S.). Lot 206 was prepared from 3 mixed breed cats, whose pelts (including hair) were rinsed in water, dried unfrozen under vacuum, cut into strips, defatted with ethyl ether, and extracted with water 1:10 w/v. The extract was dialyzed against water and freeze-dried with 1% mannitol. Lot 307 was also prepared from 3 cats; their pelts were scrubbed on both sides using a brush and water. Scrubbed pelts were dried unfrozen under vacuum, defatted by homogenizing in a blender with acetone, extracted with water 1:10 w/v, and freeze-dried with 1% mannitol. Lot 206 had 592 μ g of protein per vial by Lowry and 560 μ g of protein per vial by Kjeldahl nitrogen; an average value of 576 μ g protein per vial was used. Lot 307 had 416 μ g protein by Lowry and 435 μ g protein per vial by Kjeldahl nitrogen; the average value of 426 μ g protein per vial was used.

Saliva was collected from 3 cats (obtained from the Veterinary Resources Branch, NIH), which were given 0.2 ml of intramuscularly of 2% pilocarpine HCl (ophthalmic, Cooper Laboratories, San German, Puerto Rico) to induce salivation. Thirty to 40 ml of saliva were collected as it flowed over the mouth. Saliva from serial collections was stored at -30°C , thawed, pooled, freeze-dried, and stored at -30°C . Fifty milligrams of freeze-dried material was rehydrated with 5 ml of deionized water and found to contain 1.5 mg of protein per milliliter. This preparation was used in the Ouchterlony, radialimmunodiffusion (RID),² radioallergosorbent test (RAST), and crossed immunoelectrophoresis (CIE) analyses. Blood was obtained by cardiac or venipuncture, and the serum was separated, pooled, and stored at -30°C . Serum contained 73.7 mg protein/ml and was diluted 1:10 for all analytical procedures. Urine, collected from several cats by bladder puncture, was pooled, dialyzed against distilled water in tubing designated to have a 3500-D exclusion limit (Spectrapor No. 3, Spectrum Medical Industries, Inc., Los Angeles, CA) and stored at -30°C . This urine preparation was found to contain 500 μ g of protein/ml.

Cats were anesthetized with ketamine HCl (Parke-Davis, Detroit, MI) at 10 mg/lb intramuscularly for all of the above procedures. Collection of serum, saliva, and urine was kindly performed by Drs. Norman R. Hayes and Karen Wright, Division of Research Services, Veterinary Resources Branch, NIH.

Cat allergen 1 was obtained on several occasions from Dr. John Ohman. A highly purified preparation was used to immunize sheep for the production of monospecific anti-allergen 1 serum for Ouchterlony and RID tests. Globulin prepared from this serum showed 1 precipitin band with whole pelt extracts and saliva and no bands against serum using CIE.

Since the initial preparation of allergen 1 was consumed in animal immunizations and some preliminary studies, 2 other preparations from Dr. Ohman were also used: a whole pelt extract (No. 149) designated to contain 15.5 μ g/ml of allergen 1, and a purified allergen 1 preparation containing 10 μ g/ml.

Human allergic sera. A large serum pool (2 liters) was obtained from Drs. G. L. Gleich and J. W. Yunginger of Mayo Foundation and was labeled pool 1. It was obtained from 5 untreated individuals, each of whom had clinical symptoms of cat allergy and had positive skin and RAST tests. Pool Grater, from Dr. William B. Grater of Dallas, TX, was prepared from 3 sera, each obtained in too small a quantity to be used individually. Pools 2 and 3 were prepared from 5 and 2 individuals, respectively, each serum obtained in small quantity from Dr. S. Bukantz of Tampa, FL. One additional serum from this source was in sufficient quantity to be used alone for crossed radioimmunoelectrophoresis (CIE). Four individual sera were obtained from Dr. Richard Evans of Walter Reed Army Medical Center.

For controls, sera were obtained from each of the 2 authors, who are nonatopic, and 2 serum pools of 5 individuals each, prepared from individuals allergic to ragweed and grass pollens, respectively, and positive to these extracts by RAST.

The allergen 1 content of various preparations was measured by RID. For this procedure, 2.0 ml of a solution of 1% Noble agar containing 1% sodium azide was cooled to 55°C , mixed with 0.2 ml of the monospecific sheep anti-allergen 1, and poured onto a microscope slide. Holes, 3 mm in diameter, were punched into the hardened agar-antibody mixture, immediately filled with the test solution, and incubated for 48 hr. The slides were dipped in 10% acetic acid for 2 min, and the diameters of the precipitin circles were measured. The allergen 1 content was calculated from best-fit line of the dose-response curve generated from Ohman's allergen 1 preparation; the procedure is reproducible to within $\pm 25\%$. A standard curve was prepared using pelt 149 at several dilutions and was tested for accuracy by showing that the circle size obtained using the 10 μ g/ml solution of purified allergen 1 was on the computed line. An acetone precipitated freeze-dried cat pelt extract from Hollister-Stier Laboratories, Spokane, WA, was rehydrated with deionized water to which an equal volume of

glycerol was added. This solution, designated HSAP, contained 21 mg/ml of solids, 2.1 mg/ml protein, and 16.6 μ g/ml of allergen 1 and was shown to contain cat albumin by an Ouchterlony test.

The RAST support was prepared by mixing 5 ml of the HSAP preparation with 10 ml of borate buffer, pH 8.0, and 5 ml of dioxane suspension containing 0.33 gm of Matrex (Amicon Corporation, Lexington, MA). This mixture was rotated overnight at 4°C , washed 3 times in borate buffer, pH 8.0, and once in RAST A buffer (0.1 M phosphate buffer, pH 7.5, containing 0.2% bovine serum albumin, 5% Tween 20, and 0.01% sodium azide). The sensitized beads were resuspended in 300 ml RAST A, and 500 μ l of this support were used in each test.

RAST inhibition was carried out as described (3). Serial dilutions of each test material were prepared in RAST A Buffer, and 50 μ l of serum from a serum pool of 3 untreated cat allergic individuals were added to each tube. The support was added, and the tubes were rotated overnight at room temperature. The tubes were centrifuged, washed twice with RAST B buffer (0.1 M phosphate buffer, pH 7.5, containing 5% Tween 20), and then 25 μ l of ^{125}I -radiolabeled anti-human IgE (Atlantic Antibodies, Westbrook, ME) containing approximately 2 ng of antibody and 25,000 cpm were added to each tube with 475 μ l of RAST C buffer (0.1 M phosphate buffer, pH 7.5, containing 4% bovine serum, 5% Tween 20, and 0.1% sodium azide). The tubes were rotated overnight at room temperature, and the washing procedure was repeated before the tubes were counted in a gamma counter. The degree of inhibition of the test sample was determined by subtracting the counts bound by the sample from a positive, uninhibited control value and dividing by the difference between the positive and negative controls. To determine the potency of each preparation relative to HSAP (see Table I, column 4), a best-fit regression line was computed using the volume of preparation in serial 4-fold dilutions vs percent inhibition. The slope of the regression line was then individually compared with the HSAP regression line. For those preparations in which the differences in the slope of the lines were found not to be statistically different at $p < 0.01$, a common slope was computed. From this data, a new best-fit line was computed for the HSAP reference and the test preparation; the interval between the 2 parallel lines is the log of the relative potency. Since the slope of the inhibition response of serum was found to be statistically different from HSAP, an approximate relative potency is reported for comparison purposes.

Because of the difficulty in interpreting relative potency when expressed as microliters of samples, the relative potency RAST data was calculated as a function of the protein content (see Table I, column 5) and of the allergen 1 content (see Table I, column 6) of each test preparation.

The HSAP preparation used to make the support was also used as an inhibitor, as were preparations of serum, saliva, urine and pelts 206 and 307. All preparations were tested at their original protein concentration, except serum, which was diluted 1:10 for RAST inhibition work.

CIE and CRIE methodology has been described (4). Briefly, the test materials were electrophoresed at 10 V/cm for 30 min in 1% agarose gel at 10°C with Svendsen's buffer used as the running buffer. After the 1st electrophoresis, gel strips containing the electrophoresed antigen solutions were placed on 5 $\times$ 5 cm glass slides, and agarose-containing rabbit antibody to cat pelt extract was poured above the gel strip. A 2nd electrophoresis was then run for 18 to 20 hr at 1.5 V/cm and at right angles to the direction of the 1st electrophoresis. At the conclusion of the run, the plates were pressed, washed in normal saline, and dried.

Before staining, selected slides containing the immobilized antigen for CRIE were covered with serum from the pool of untreated cat allergic individuals. After overnight incubation with gentle rotary shaking, the plates were washed with normal saline, covered with radiolabeled anti-human IgE (0.2 μ g antibody, 375,000 cpm) and allowed to incubate overnight with gentle shaking. The plates were washed thoroughly with saline and loaded into x-ray film cassettes with Kodak Royal Blue x-ray film (Eastman Kodak Company, Rochester, NY 14650). At intervals of 4, 7, and 14 days the films were developed for the CRIE images and the slides were stained with 0.25% Coomassie Brilliant Blue R-250.

By incorporating antigens in an intermediate gel, just above the electrophoresed pelt extract, antigens or allergens that are in the bottom and intermediate gels form precipitin identity peaks or lines (4). By using allergen 1-specific globulin in the upper gel, the allergen 1 peak was identified, thereby permitting its subsequent location in patterns produced with anti-sera to whole pelt extract. By mixing an excess of an albumin-enriched material with a pelt extract before electrophoresis, the great increase in the size of the albumin peak resulted in its identification.

Total nitrogen was obtained by Kjeldahl (carried out by the Microanalytical Services Branch of the National Institute of Arthritis, Metabolism and Digestive Diseases) and multiplied by 6.25 to calculate the protein content of the pelt Lots 206 and 307. The protein content of other preparations was obtained using a BSA standard with the procedure of Lowry *et al.* (5).

Anti-cat pelt serum and globulin were obtained by immunizing 4 New Zealand rabbits (2 kg) with cat pelt extract. Globulin was prepared by precipitating serum with 25% $(\text{NH}_4)_2\text{SO}_4$ as described (4). Anti-cat albumin was prepared in rabbits by a similar immunization procedure, using a cat albumin preparation provided by Dr. John Ohman. Due to the very small

² Abbreviations used in this paper: RID, radialimmunodiffusion; CIE, crossed immunoelectrophoresis; CRIE, crossed radioimmunoelectrophoresis; RAST, radioallergosorbent test; CFA, complete Freund's adjuvant; ICFA, incomplete Freund's adjuvant, i.e., containing no mycobacteria; HSAP, Hollister-Stier acetone precipitated cat pelt extract.

quantity of pure albumin and the small quantity monospecific serum obtained, it was not possible to develop a test to quantitate the albumin.

RESULTS

Table I gives a list of preparations tested and their properties. All preparations contained protein; however, pelt 307, serum, and urine contained no detectable allergen 1. Since the concentration of protein and allergen 1 in the preparations are variable, the RAST potency (column 4) is not directly interpretable. In columns 5 and 6 the RAST potencies are corrected for protein and allergen 1 concentrations, respectively. It is apparent that the RAST potency is unrelated to the protein content of the preparations even for preparations as similar as pelts 206 and 307. From the data, it appears that allergen 1 content, rather than total protein, is most closely correlated with RAST potency. The relative potency of saliva, which is 2.44, based on its allergen 1 content, appears to be higher than that of HSAP, but the potency of the 2 materials is not statistically different at 2 SD.

The CIE pattern of cat pelt 206 with globulin prepared from rabbit anti-pelt serum (Fig. 1A) shows that there are 8 detectable antigens numbered in the schematic drawing (Fig. 1B). The CRIE pattern, derived from Figure 1A by radioautogram, is shown in Figure 1C, with a schematic drawing in Figure 1D that shows 2 strong bands and 2 weak ones. The allergen 1 band (band 1, see below for identification) is the most intense and becomes visible in less than 4 days. The albumin peak (band 2, see below for identification) appears later, and the faint peaks are visible only after 10 or more days.

To determine the distribution of IgE antibodies to the various allergens, CRIE was also carried out with the Mayo serum pool; Grater serum pool, pools 2 and 3 as well as the single serum from Dr. Bukautz, and the 4 sera from Dr. Evans. Controls consisted of the sera from the 2 nonatopic authors and the 2 serum pools from individuals allergic to ragweed and grass pollens. All of the sera from cat allergic individuals contained IgE to allergen 1 and, with 1 exception, allergen 1 was the most intense band in the radioautograms. All of the sera also contained IgE to albumin, but generally this took several additional days to develop in the radioautograms. They also showed one or more additional very weak bands in the radioautograms. The 2 sera from nonatopic individuals and the 2 serum pools from pollen-allergic individuals showed blank film in the radioautograms.

Figure 2A shows a CIE of pelt 206 using anti-cat-pelt globulin, but in Figure 2B, anti-cat-pelt was replaced with anti-cat-allergen 1 globulin. This results in the identification of the allergen 1 peak and establishes that this allergen is not detectable in the CIE of pelt 307, as seen in Figure 2C. Figure 2D shows a light load of 307 mixed with a cat albumin preparation before electrophoresis. The greatly enhanced peak identifies the cat albumin band. These modifications of CIE techniques confirm the results of the RID

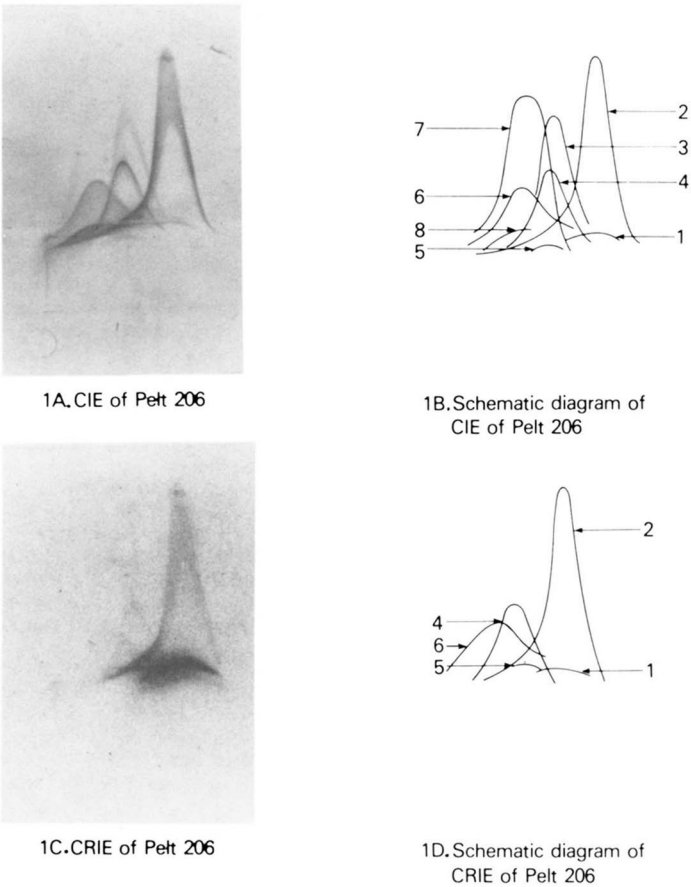


Figure 1. CIE and CRIE patterns of pelt 206.

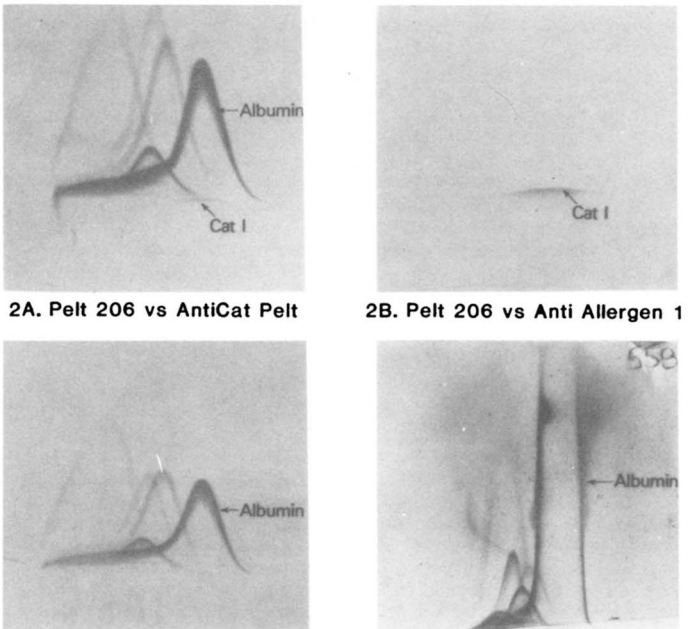


Figure 2. Identification of allergen 1 and albumin by CIE.

TABLE I					
Properties of cat allergen preparations					
Preparation	Protein	Allergen 1	RAST Potency		
			Relative to HSAP	Relative ^a to HSAP protein	Relative ^b to HSAP allergen 1
	μg/ml	μg/ml			
HSAP	2100	16.6	1.00	1.00	1.00
206	576	2.7 ^c	0.17	0.63	1.06
307	426	0 ^c	0.015	0.069	
Saliva	1500	10.3	1.5	2.1	2.4
Serum	7370	0	0.072 ^a	0.022	
Urine	500	0	0.013	0.055	
Allergen 1	10	10.0	0.93	196	1.6

^a Relative potency to HSAP protein
= $\frac{\text{RAST potency relative to HSAP (col 4)}}{\text{Protein of prep/protein of HSAP}}$

^b Relative potency to HSAP allergen 1
= $\frac{\text{RAST potency relative to HSAP (col 4)}}{\text{Allergen 1 of prep/allergen 1 of HSAP}}$

^c Minimal detectable level of allergen 1 by RID is 4 μg/ml, therefore, preparations reconstituted to 500 μl (1/2 required volume) for analysis.

^d The slope of the inhibition line of serum was statistically different from HSAP, consequently, the relative potency is approximate.

analysis in Table I in demonstrating that allergen 1 could not be detected in 307.

Urine, serum and 307 have very low but roughly similar allergenic potencies (Table I), but these materials, as well as saliva and HSAP (Fig. 3), have albumin.

To examine the antigenic and possible allergenic differences between pelt and saliva, a CIE pattern (Fig. 4) obtained by the electrophoresis of Lot 206 was followed by electrophoresis in the

2nd direction through saliva placed in the intermediate gel into the upper gel containing anti-cat-pelt serum. This experiment demonstrated all of the pelt antigens of 206 (with the possible exception of 1 very faint one) are moved upward, showing that the antigenic composition of saliva and pelt are almost identical.

DISCUSSION

It has been known that saliva of cats could induce an allergic response (6). Nevertheless, extracts for diagnosis and therapy are

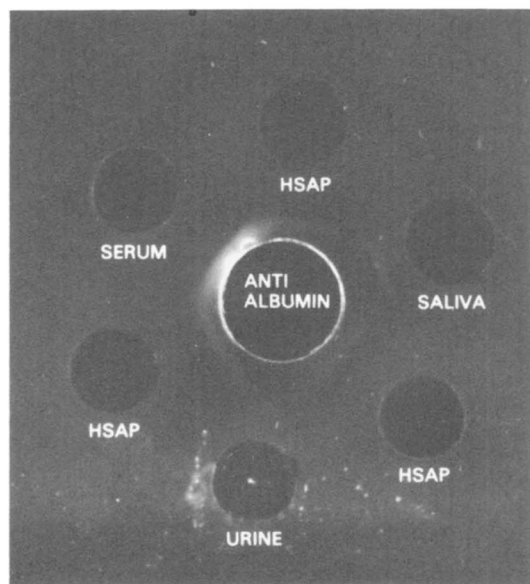


Figure 3. Ouchterlony gel diffusion of various preparations illustrating the presence of cat albumin.

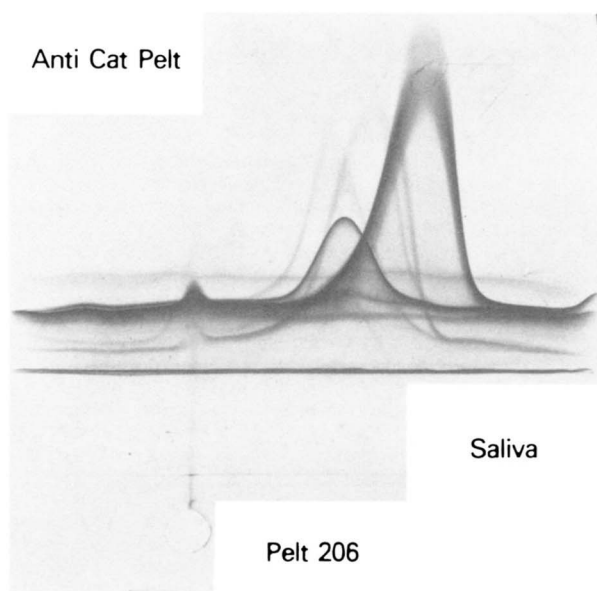


Figure 4. CIE pattern of pelt 206 with cat saliva in intermediate gel.

prepared from hair-skin-pelt and are included in the class of allergens called "epidermals." The use of pelt and hair has recently been shown to be inappropriate as an allergen in the study of rat and mouse allergy, where most of the allergenic activity was in urine (7). In the case of cat allergy, pelt extracts clearly are allergenically active, but the study of Spain *et al.* (8) demonstrated that all of the allergenic activity in the sera of cat-allergic individuals could be neutralized by saliva as well as pelt extract. Since cats lick themselves so extensively, it may be assumed that pelt allergens are not pelt derived but are adventitious substances. This possibility is compatible with the difference between pelt Lots 206 and 307, where some extra washing of the pelt before processing removed most of the allergenic activity of 307 as well as detectable allergen 1. Allergen 1 has been described and claimed to be the cat allergen that correlates with skin reactivity (2). The presence of albumin in all of the preparations would support its potential importance as an allergen, but CRIE testing with other serum pools and individual sera did not show albumin to have any higher allergenic activity than with the original serum pool. The low RAST potency of serum, given its high albumin content, also would suggest the minor importance of cat albumin as an allergen. This study confirms the earlier findings that allergen 1 is the most important cat allergen, and correlates with the RAST activity of a variety of preparations. Our findings also confirm the findings of Spain *et al.* (8) that all of the allergenic activity of pelt extracts is present in saliva, and extends those findings by demonstrating that all of the antigens of a pelt extract are also present in saliva, indicating that this may be the true source of cat allergens. The apparent ease with which allergen 1 can be removed from pelts by simple washing and the high concentration of allergen 1 in saliva tends to confirm this concept and suggests that the allergen 1 content of pelts is the result of the buildup of dried saliva. Saliva, therefore, may be the best source material for cat allergen production, which would reduce the need for large numbers of pelts and enable the same animals, whose health could be readily monitored, to be used repeatedly as a source of allergen.

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