

Our task was to expose the rats kept in a suitable chamber to cigarette smoke and to determine possible changes in the number of the goblet cells in the trachea using the method described by Reid. The aim of these tests was to determine, under Project Conqueror, whether goblet cell proliferation is in any way related to the ciliastatic activity of different cigarette samples.

3. Test Setup

3.1 Exposure Device

The exposure device built by us consists of plexiglas 5 mm in thickness. Its dimensions (length, width, height) are 59 x 41.5 x 41.5 cm. The device is divided into four chambers of equal size by partitions of plexiglas. The front wall of the device can be removed. Strong spring clips and a seal glued onto the edges of the chamber ensure a tight fit. Interchange of air between the four chambers is impossible.

In the two right-hand chambers (smoking chambers) the rats are exposed to the cigarette smoke. Each of these two chambers has two openings in its rear wall: one for the cigarette holder and one for connecting the pressure gauge and the flowmeter. The two left-hand chambers (control chambers) are provided for the control animals; they have only one opening in the rear wall for connecting the pressure gauge and the flowmeter. In addition, each of the four chambers has an opening in the front wall. They serve for connecting the hoses through which equal volumes of air can be sucked through the four chambers within equal periods of time by means of two diaphragm pumps connected in parallel.

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3.2 Exposure of the Rats

After the rats had been placed in the chambers, they were allowed to get accustomed to their environment for about half an hour. During this period of time a moderate current of air passed through the chambers, without any drop in pressure. Then, all openings of the control chambers were closed. The hoses to the flowmeter of the two smoking chambers were closed as well. After one cigarette had been put in each holder, the pressure in the smoking chambers dropped. The cigarettes were lit when the negative pressure was about 5 cm of water. Then they were smoked continuously during 4 to 5 minutes to a butt length of about 15 mm.

After smoking of the cigarettes the two smoking chambers were filled with smoke to such an extent that the rats could no longer be seen if one tried to look through the chambers in longitudinal direction. The openings of the two control chambers were opened again, the lumen of the cigarette holder was closed, and the clamp between the smoking chambers and the corresponding flowmeter was removed. Now, a continuous current of air, which was controlled by the two flowmeters, was again sucked through the four chambers. By means of the two pressure gauges it was possible to check whether the chambers were tight. To this end it was only necessary to interrupt the respective hose connection between flowmeter and pressure gauge.

About 30 minutes after smoking, the amount of smoke left in the smoking chambers was hardly visible. After another 30 minutes had passed, one further cigarette per smoking chamber was smoked, and so on, until the rats had been exposed to a maximum of eight cigarettes per day. The total exposure times for the individual tests are given in section 4.2.

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3.3 Histochemical Preparation of the Goblet Cells

The preparation of the goblet cells involved greater difficulties than had been expected because of the varying hardness of the trachea material (cartilage and ciliated epithelium). Since a histological section usually is satisfactory only if the paraffin has the same hardness as the tissue to be cut, and since good sections are a prerequisite for a reliable count of the goblet cells, we had first to try a number of fixing and embedding methods. Fixing in Bouin solution proved to be satisfactory.

In the beginning we extirpated the trachea after the animals had been killed, whereupon it contracted. In order to exclude this effect which is one of the causes of the repeatedly observed detachment of the epithelium, the trachea was extirpated, but during fixing it was attached in its in-situ length to a cork support. In order to prevent touching the trachea at all prior to fixing, it was fixed in situ with the surrounding tissue of the neck.

In the course of the tests it was found that dehydration of the tissue has to be effected very slowly and very carefully, in order to prevent detachment of the epithelium. Embedding was done in paraffin, to which different quantities of bees wax were added. However, in spite of all care it was not possible in the case of each trachea to obtain optimum sections in which the whole epithelium was undamaged. This applies also to the last test.

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For staining of the goblet cells, the sections 7 to 10 μ in thickness were subjected to the PAS reaction and, in addition, stained with Alcian blue. The PAS reaction is specific for vicinal hydroxyl, and vicinal hydroxyl and amino radicals. Therefore, polysaccharides, neutral mucopolysaccharides (MPS), muco- and glycoproteins as well as glycolipids were stained. Since the mucus of the goblet cells, however, consists partly of acid MPS, the sections were in addition stained with Alcian blue, since this is a fairly specific stain for acid MPS. In numerous sections it was found that part of the mucus was stained purplish red (PAS), while the other, mostly larger part was stained blue green (Alcian blue). If a specific section did not show goblet cells, successful staining could be checked at the cartilage (chondroitin sulphuric acid), which had to be stained satisfactorily in any case.

3.4 Goblet Cell Count

From the middle piece of each trachea six to eight sections were prepared (no serial sections), which were mounted on one slide. These sections were first observed at low magnification. The section showing the best-preserved epithelium was selected for counting the goblet cells. Counting was done at high magnification (1.000 X). The preparation was adjusted such that the epithelial layer was in the middle of the field of view. Thus it was ensured that the epithelium always had the same length. All goblet cells visible in 20 high power fields were counted. From the total number of the goblet cells counted in this way, the mean value per high power field was calculated. This figure was regarded to be representative of the trachea of the rat concerned.

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4. Results

4.1 Behaviour of the Rats during and after Smoking

On the first and second day of a test the rats were exposed to the smoke of only half-smoked cigarettes at intervals of one to two hours. In this way they got quickly adjusted to the conditions described in section 3.2, which the majority of the rats tolerated without visible signs of disease. Only if the cigarettes were smoked within less than four to five minutes, as was done several times in the beginning, so that the chambers were filled with very large amounts of smoke, heavy spasms were observed. It is to be assumed that these were rather due to a lack of oxygen than to nicotine poisoning.

It was observed that the respiratory frequency of the exposed rats was higher than that of the controls for a certain period of time, even if there was no longer any smoke in the chambers.

After six to eight days' exposure, some of the rats showed a nasal breathing-sound and, in addition, slight nasal discharge. Their food consumption, however, remained normal.

The rats mostly slept during their stay in the chambers. As soon as they had been put back into the Macrolon cages after the exposure, they started cleaning their fur. The fur of the rats exposed to the smoke was stained brown after several days' of exposure already.

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4.2 Results of the Goblet Cell Count

Test 1: Cigarettes Type 4CSS22, 6 Days, 32 Cigarettes

The first test was carried out on eight male Sprague-Dawley rats (200 to 250 g) which were exposed to the smoke of 32 cigarettes of the highly irritating type 4CSS22 for six days. During the last three days of the test, the rats were exposed to the smoke of eight cigarettes per day. The animals were sacrificed 14 days after the last exposure. The controls, eight in total, belonged to the same strain and were of the same age.

It was not possible to evaluate preparations of more than three of the exposed animals and five of the controls, since in the remaining preparations the epithelium was largely detached or torn. Repeated sections likewise proved unsatisfactory.

Table 1: Average Goblet Cell Count
per High Power Field and Rat

Controls	4	8	2	1	1	-	-	-
Exposed	11	15	19	-	-	-	-	-

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Test 2: Cigarette Type 4CSS22, 7 Days, 57 Cigarettes

The second test was carried out on eight female rats of a Wistar strain on eight successive days. The rats were exposed to the smoke of 57 cigarettes in total; the cigarettes were again of the highly irritating type 4CSS22. Eight rats of the same kind served as controls. In this test, all rats were sacrificed immediately after the last exposure.

It was not possible to evaluate the preparations of three of the controls because of their unsatisfactory quality.

Table 2: Average Goblet Cell Count per High Power Field and Rat

Controls	9	5	1	1	1	-	-	-
Exposed	16	13	5	17	19	8	11	10

Although the results of all the five tests will be discussed in the next section, we should like to mention here that in this test for the first time less goblet cells were found in an exposed animal than in the controls.

Test 3: Cigarette Type 4CSS22, 15 (13) Days, 99 Cigarettes

Eight female rats (180 to 200 g) of the Wistar strain which had also been used in Test 2 were exposed to the smoke of a total of 99 cigarettes of type 4CSS22 for 15 days. The exposure was interrupted for two days on a weekend. Again, eight rats served as controls. All animals were sacrificed immediately after the last exposure.

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Table 3: Average Goblet Cell Count
per High Power Field and Rat

Controls	5	7	7	3	1	2	3	6
Exposed	7	5	10	15	15	14	13	14

Test 4: Cigarette Types 4CSS22 and R305, 17 (13) Days,
100 Cigarettes

This test was carried out on 18 female rats (9 of which served as controls) of the same weight class and the same strain as in Test 3. Since only 67 cigarettes of type 4CSS22 were left, the test was concluded with cigarettes of type R305. On two weekends of the test period, the rats were not exposed. They were again sacrificed immediately after the last exposure. Preparations taken from three of the exposed animals did not permit evaluation.

Table 4: Average Goblet Cell Count
per High Power Field and Rat

Controls	4	1	1	3	5	4	1	4	2
Exposed	12	7	7	5	10	11	-	-	-

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Test 5: Cigarette Type R305, 10 Days, 88 Cigarettes

Eight female rats of the Wistar strain (120 to 150 g) were exposed to the smoke of 88 cigarettes R305 on ten consecutive days. Six rats served as controls. The animals were sacrificed 24 hours after the last exposure.

In this test, again, the preparations of three animals were unsuitable for evaluation because the ciliated epithelium was largely detached.

Table 5: Average Goblet Cell Count
per High Power Field and Rat

Controls	2	3	2	3	3	-	-	-
Exposed	10	8	5	5	5	6	-	-

5. Discussion of the Results of the Goblet Cell Count

Although the results of the goblet cell count of the five tests are comparable with each other with some reservation, we should like to start this discussion with the following synoptic table:

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Table 6: Summary of Results of the Goblet Cell Count

Test No.	1	2	3	4	5	
Exposure time, days	6	7	13	13	10	
Cigarette sample	4CSS22	4CSS22	4CSS22	4CSS22 R305	R305	
Number of cigarettes smoked	32	57	99	100	88	
Average goblet cell count	Controls	3.3	3.6	4.2	2.8	2.6
	Exposed	15.0	12.4	11.6	8.6	6.5

Please note that the values indicated for the average goblet cell count are applicable only in conjunction with the individual values of Tables 1 to 5 which, in part, scatter widely. Also, the number of individual values per average value differs. Still, we believe, that these values permit the following inferences to be made:

1. The tests have shown that indirect exposure to cigarette smoke results in an increase in the number of goblet cells in the trachea of rats.
2. The results obtained support the assumption that the smoke of highly irritating cigarettes increases the number of goblet cells to a greater extent than does smoke of less irritating cigarettes.

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3. The results of the first two tests suggest that even a brief exposure of the animals to cigarette smoke is sufficient to obtain an increase in the number of goblet cells.

The following aspects are essential in the further interpretation of the findings:

A striking result is the difference in the figures obtained in some cases for the average goblet cell count per high power field in the controls and the exposed animals. This may be explained not only by biological variation, but also by methodical errors as indicated in the following:

The goblet cells extend from the basal membrane to the surface of the epithelium. They are filled with mucus according to their functional state. It always depends on the plane and thickness of the section whether a goblet cell is visible in its entire length, only partly or not at all. In an ideal section the epithelium is cut so as to contain both basal cells and the cilia. This is the only case where the number of goblet cells can be determined with certainty. Although this aspect has been considered in the goblet cell count, not all preparations which were evaluated showed the epithelium in an ideal section. If the studies are to be continued systematically, this source of error can be excluded by counting the goblet cells not just of one section but of numerous (about 10) serial sections.

The necessity to evaluate serial sections of several parts of the trachea is further substantiated by the fact that the goblet cells are not uniformly distributed in the mucous membrane. No doubt, this is part of the reason why, in many controls, only individual goblet cells have been found.

Apart from these techno-histological and statistical sources of error, the great differences in the shape of the goblet cells are important for interpreting the results. The goblet cells of rats rarely ever have goblet shape. Normally, they are more or less wide columns of stainable material. In their

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distended state only, i.e. when storing mucus, are the cells of goblet shape. Distended goblet cells have been found in controls and exposed rats alike. However, if a preparation contained an average of 15 or more goblet cells per high power field, the cells were never distended. They rather represented very narrow columns of stainable material, often containing but a few, faintly stained granules. The difference in the shape of the goblet cells has not been considered in the present findings. Thus, a rat in which only five or seven goblet cells were found per high power field despite the exposure (Test 3), may well have produced the same amount of mucus than a rat with ten or more goblet cells. The figures of the goblet cell count are nonconclusive in this respect. In future tests it would be necessary to make a (semiquantitative) statement on this point, in addition to indicating the number of goblet cells.

Summarizing, we should like to point out that in our opinion the results of the preliminary experiments justify the continuation of these investigations. The studies would have to be conducted according to a systematic plan aimed at a possible correlation between the proliferation of goblet cells and the ciliastatic activity. If this objective is desired we shall prepare a detailed proposal on the basis of the experience and knowledge gained so far. We shall be in a position to continue the tests by April or May 1966.

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