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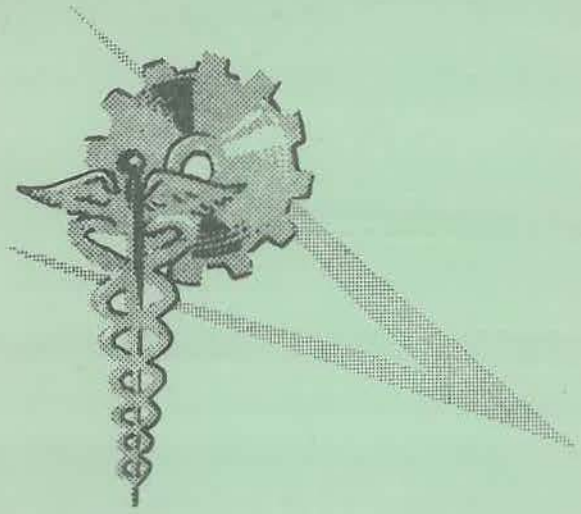

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Public Health Service
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SUMMARY

Mice, rats, and rabbits quickly acquire a tolerance to ozone when given a single nonlethal exposure prior to challenge. Rabbits which have been exposed to ozone have been reported to develop precipitating antibodies against ozonized egg albumin. The question has been raised whether immunochemical phenomena are responsible for the acquired tolerance. Studies reported here are intended to help resolve the question. They are based on two considerations: (1) experimental observations by others indicate that, in general, serum glycoproteins increase during the course of development of immunity and (2) the ability of the agar diffusion technique to indicate identity or non-identity of precipitating antigen-antibody reactions. Our results using both approaches were found not to support an immunochemical hypothesis for the development of tolerance to ozone in rabbits.

INTRODUCTION

Stokinger, Wagner, and Wright, using rats[1], and Svirbely and Saltzman, using rats and mice[2], observed a tolerance-producing effect of ozone. One nonlethal exposure to ozone rapidly produces a tolerance in these animals to a succeeding acute exposure which exceeds the quantity normally required to produce pulmonary edema and death. A tolerance to multilethal doses was produced in rats 1 day to 4 weeks after one 6-hour exposure to 1 ppm ozone by Stokinger[3]. On autopsy Stokinger's tolerant rats showed no pulmonary edema or hemorrhage, whereas these changes always occurred in the ozone-challenged nontolerant controls. Matzen verified previous studies with mice and raised the question of a possible relationship between tolerance and an immune reaction[4]. Precipitating antibodies have been claimed to be demonstrable in the serum of rabbits exposed to

ozone[5]. A concept involving a specific immune reaction in production of tolerance is obscured by the production of a cross tolerance between the two oxidant gases ketene and ozone, shown by Mendenhall and Stokinger[6]. Further, Henschler demonstrated that the nonoxidant gas phosgene produces a tolerance against the oxidant gases NO_2 and O_3[7]. On this basis Henschler concluded that an antigen-antibody phenomenon appeared to be excluded since the protective action was not specific for a definite class of gas.

The present work provides further information to aid in the assessment of the immunochemical hypothesis. It is based on two approaches: (A) the work of Weimer, et al.,[8,9,10] Hammerstrom[11], and others, which has indicated that, in general, serum glycoproteins are elevated as immunity develops; and (B) the sensitive agar double-diffusion technique of Ouchterlony[12] which was used to determine whether ozonized egg albumin produced different precipitating antibodies in rabbit serum from those produced by nonozonized egg albumin[5].

METHODS AND PROCEDURES

SERUM GLYCOPROTEINS

Five to seven milliliters of blood was taken via marginal ear vein from each of 8 male New Zealand white rabbits of 2.5 - 3.5 kg weight. The blood was chilled, centrifuged, and the serum frozen at -20°C until analyzed for total serum polysaccharide (representing total glycoprotein) and serum mucoprotein polysaccharide (representing mucoprotein) according to Weimer[13]. On the same day, four of these rabbits were exposed to ozone for 40 minutes. The C.T. (concentration in ppm x time in hours) was 10.3 ppm-hours. Ozone concentration was determined according to Byers and Saltzman[14].

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Immediately after exposure of the four rabbits, all eight rabbits were again bled and the serum preserved as above. The following day, and for a total of 14 consecutive days, all rabbits were bled and their sera kept at -20°C until analyzed.

A 5-week study of total serum glycoprotein polysaccharide and serum mucoprotein polysaccharide was performed on another group of five rabbits, separately exposed once, to an average ozone concentration of 10-12 ppm for a period of approximately 1 hour. The C.T. value varied from 10.5 - 17.6 ppm-hours. Pre-exposure and immediate post-exposure samples of blood were taken. Samples were then secured weekly for a period of five weeks. Serum total glycoprotein analyses were performed in duplicate from aliquots of the same serum sample. Owing to limited amounts of material the serum mucoprotein polysaccharide values were obtained on single aliquots of blood specimens in all cases.

In evaluating the results, the percent deviation of each value from the mean for each rabbit was used in making comparisons. To determine whether alterations in the mucoprotein and in the remaining glycoprotein were concomitant, or whether they varied separately, the mucoprotein polysaccharide values were subtracted from the corresponding total glycoprotein polysaccharide values to give a remainder termed T-M (Table 1).

To test the data, linear regressions of concentration on time were computed and the slopes subjected to standard t-tests. Results of such tests are given in the table. A probability of at least 95% that there was a regression of concentration on time was considered real. Also, when two regression lines were compared, a probability of at least 95% that they were different was considered real.

AGAR DIFFUSION

Rabbits used in these experiments were of the same type and size as were used in the previous section. The entire procedure was carried out twice. A 2.3×10^{-5}M solution of commercial 3x crystallized

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hen egg albumin in N saline was prepared and divided into two equal portions. To each portion was added a small drop of silicone anti-foam. One portion (Ea) was placed in the deep freeze and ozone in air at 20°C was bubbled through the other portion at 0.5 liter per minute for 12 minutes. Ozone concentration before entering the solution averaged about 7.1 ppm while an excess of about 1.5 ppm passed through. A total of approximately 5-5.4 moles of ozone per mole of protein was retained by the solution. Whether or not this amount of ozone reacted directly or indirectly with the egg albumin is not known. However, some of the protein precipitated and some remained in solution. This entire material is called ozonized egg albumin (OEa). A part of both OEa and Ea were precipitated with alum[15]. Aliquots of the well-shaken suspensions of alum precipitated OEa and Ea were used to give a course of injections to rabbits. Two rabbits not exposed to ozone, were used for each material which was injected daily via a marginal ear vein in amounts increasing from 0.35 ml to 1.0 ml over a period of 12 days. The following sera were used in the agar diffusion technique: (1) from the rabbits injected with OEa, (2) from those injected with Ea, (3) from a rabbit which had been exposed 5 days per week for 6 months to 1 ppm ozone, (4) from two rabbits which had been exposed 4 days prior to use to 12.3 ppm ozone for 1 hour. Agar diffusion plates were prepared according to Ouchterlony[12] using a 4-cup procedure with each possible combination of diagonally and directly opposed antigen and serum in the cups.

RESULTS AND DISCUSSION

SERUM GLYCOPROTEINS

The following facts are observed in the Table: (1) A diminution occurs in the serum mucoprotein polysaccharide and in the remaining polysaccharide (T-M) in rabbits bled immediately before and after one exposure to ozone for

10.3 ppm-hours, and bled each day for 14 days thereafter. (2) A diminution occurs in the serum mucoprotein polysaccharide, but not in T-M, in control rabbits bled each of 14 days. (3) A significant difference exists between the slopes of the regression lines of the T-M fraction of exposed rabbits bled each day for 2 weeks and that of the same fraction in sera of ozone-exposed rabbits bled once each week for 2 weeks. (4) A similar test comparing control rabbits bled each day to exposed rabbits bled each week shows a significant difference in the slopes of the regression lines. One exposure to ozone does not produce a more significant drop in the T-M fraction, when rabbits are bled each day, than bleeding alone produces. Finally, since rabbits exposed once to ozone and bled weekly for 2 weeks or 5 weeks do not show significant increase in either of the measured glycoprotein components, these results do not lend support to an immunochemical hypothesis for the acquired tolerance which results from such exposure.

AGAR DIFFUSION

Undiluted sera from each rabbit was first overlaid with the soluble part of OEa and separately with Ea solutions. An immediate and heavy precipitin ring formed at the boundary in each case when the serum came from a rabbit that had been immunized against eighter OEa or Ea. This result could be interpreted as meaning that the OEa contained Ea or that OEa and Ea produced identical or cross-reacting antibodies.

The sera from neither the long-term nor the briefly-exposed rabbits reacted with either of the egg albumin preparations in the 0.5 hour permitted for the precipitin rings to develop. However, upon standing for several hours, a faint ring appeared in each test which could be attributed to adsorption of precipitated OEa or Ea at the boundary and not necessarily associated with antigen-antibody combination[5].

In attempts to resolve this question, the agar double-diffusion technique was used to test for identity of antibodies developed in rabbits against OEa or Ea. In each of many experiments all precipitin lines united into a single front without visible trace of overlap for the duration

of the experiment (one week). No additional precipitin lines were formed by the OEa vs anti-OEa serum which again indicates that if the OEa contained Ea, the antibodies formed were either identical or completely cross-reacting within the limits of sensitivity of the method. The same technique was also used to see if precipitin lines might be formed when the sera of the long-term and short-term exposed rabbits were tested against OEa and Ea. The results were negative after one week in each case. These results may be compared with those of Seligman[16], who found that degradation products of fibrinogen and fibrin could not be distinguished immunologically when allowed to react with antifibrinogen immune serum in the ring test or the Ouchterlony double-duffusion technique.

At present, therefore, any immunochemical hypothesis relating antibody formation and production of tolerance to ozone in experimental animals should incorporate the corollary of formation of widely heterogeneous precipitating antibodies with identical agar diffusion characteristics or nonprecipitating antibodies, either of which are produced by both oxidant and non-oxidant gases without an attendant increase in serum glycoproteins.

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TABLE 1

t-Test Summary

Daily Bleeding	Slope of Regression Line	Probability
T-M*		
Exposed (A)**	-0.192	> 95
Control (B)	-0.219	> 90
Muco		
Exposed (C)	-0.436	> 95
Control (D)	-0.661	> 99
Weekly Bleeding		
2 Weeks		
T-M (E)	+1.95	80
Muco (F)	+2.11	60
5 Weeks		
T-M (G)	+0.581	80
Muco (H)	+0.148	50
	Comparison of Slopes	
(A) vs (E)		> 98
(B) vs (E)		> 95
(A) vs (B)		50
(C) vs (F)		85
(E) vs (F)		50
(G) vs (H)		50

* T-M is the test abbreviation for total serum glycoprotein minus serum mucoprotein.

**The letters (A)--(H) facilitate comparison of slopes at bottom of table.