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CIGARETTE SMOKE—A PROPOSED METABOLIC LESION IN ALVEOLAR MACROPHAGES*

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Abstract—The effects of the filtered gas phase of cigarette smoke were investigated with respect to certain enzyme activities of rabbit alveolar macrophages. Preliminary experiments with crystalline preparations of glyceraldehyde 3-phosphate dehydrogenase showed that cigarette smoke is a potent inhibitor of this enzyme and that protection from inhibition by smoke is afforded by cysteine. Other experiments, in which enzyme activity was measured in alveolar macrophages, demonstrated inhibition of glyceraldehyde 3-phosphate dehydrogenase activity in cells treated with cigarette smoke and protection by cysteine. There was no significant impairment of the activities of glucose 6-phosphate dehydrogenase and lactic dehydrogenase in smoke-treated macrophages. Histochemical evidence supported these findings. It is suggested that a relationship exists between loss of alveolar macrophage phagocytic competence and inhibition of glyceraldehyde 3-phosphate dehydrogenase. This paper discusses impaired phagocytosis by macrophages in the presence of cigarette smoke in relation to concomitant reduction in glyceraldehyde 3-phosphate dehydrogenase activity.

CIGARETTE smoking has been implicated as extremely undesirable with respect to healthy pulmonary function and much attention has been focused on various aspects of lung function and disease in cigarette smokers. In particular, it has been demonstrated,[1] in mice, that clearance of bacteria by the lung is markedly decreased after exposure to cigarette smoke and Ferin, Urbankova and Vlekova[2] showed that the dynamics of lung clearance of inhaled silica particles, in rats, can be affected by modifying the functional activity of macrophages by exposure to tobacco smoke.

Alveolar macrophages may be considered as the first line of pulmonary defense and occupy a functional position of primary importance in pulmonary clearance since they are capable of the uptake, transport and elimination of particles, microorganisms and fluids through the processes of phagocytosis, pinocytosis and digestion. Impairment of these activities may thus have serious consequences with respect to the defense mechanisms of the lung. In spite of the known hazards of cigarette smoking to the lung, it is only comparatively recently that the function of the alveolar macrophages in the presence of cigarette smoke has received attention. Green and Carolin[3] have reported experiments which demonstrate that the presence of cigarette smoke or the filtered gas phase of cigarette smoke has a marked inhibitory effect with respect to the phagocytic function of rabbit alveolar macrophages *in vitro*.

Although the toxic effect of cigarette smoke with respect to the functional activity of macrophages has been documented[3,4] there is little information on the biochemical

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lesions underlying the physio-pathological changes. The present study seeks to investigate the enzymological basis for impairment of phagocytic function resulting from exposure of the cells to smoke.

Glycolysis has been implicated as the energy source for phagocytosis in polymorphonuclear leukocytes[5–7] but the contribution of the glycolytic pathway to the phagocytic event in alveolar macrophages is less well understood. Comparative studies on phagocytosing cells[8] have shown alveolar macrophages to depend heavily on cytochrome-linked respiration for particle ingestion since interference with aerobic metabolism or oxidative phosphorylation had a depressive effect on particle uptake. It was, however, observed that inhibition of glycolysis was even more damaging to phagocytic ability, indicating perhaps that the cells are not entirely dependent on oxidative phosphorylation but can perform the phagocytic event, to some extent, by energy provided by glycolysis. In this connection, Green (unpublished data) has shown that there is some reduction in phagocytic competence under anaerobic conditions but the extent of the inhibitory effect of cigarette smoke is much greater and thus consistent with an effect on anaerobic metabolism.

It has been shown that the phagocytic competence of polymorphonuclear leukocytes is markedly impaired when the sulphydryl reagent, iodoacetate, is employed as a metabolic inhibitor.[7] Although investigations studying the effects of iodoacetate on phagocytosis do not directly implicate glyceraldehyde 3-phosphate dehydrogenase, it is probable that this enzyme would be most susceptible and its inhibition would result in a block in the glycolytic pathway. In this context, it is particularly noteworthy that Green[4] has reported that rabbit alveolar macrophages could be protected from the effects of cigarette smoke by the thiols, cysteine and glutathione. Collectively, these observations may imply a sulphydryl role for cigarette smoke and further suggest that impairment of the glycolytic pathway may be responsible for the observed functional defect. It seemed possible that, in macrophages, the biochemical lesion may result from modification of SH groups of glyceraldehyde 3-phosphate dehydrogenase in the presence of cigarette smoke. The experiments reported here were designed to test this hypothesis by demonstrating specific inhibition of glyceraldehyde 3-phosphate dehydrogenase by the filtered gas phase of cigarette smoke (a) in alveolar macrophages by histochemical staining methods, (b) in purified crystalline enzyme preparations, and (c) in extracts of lysed alveolar macrophages.

MATERIALS AND METHODS

Materials. NAD, NADH, NADP, tris, DL-glyceraldehyde 3-phosphate diethyl acetal barium salt, glucose 6-phosphate, 3-phosphoglyceric acid, bovine serum albumin, ATP, crystalline rabbit muscle glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglyceric acid kinase were all obtained from Sigma Chemical Co., St. Louis, Mo.

Cigarette smoke. Freshly drawn filtered cigarette smoke was used in all experiments and was prepared as described by Green and Carolin.[3] A plastic 50-ml syringe was fitted with a 3-way stopcock and a Cambridge filtering device (Phipps & Byrd). The end of an unfiltered cigarette was inserted into a short piece of polyethylene tubing attached to the filtering device and "smoking" consisted of adjusting the stopcock so that a puff could be drawn into and subsequently expelled from the syringe. One 35-ml puff (drawn in 2 sec) was drawn every minute for 7 min and the

seventh puff was retained by closing the stopcock. In experiments using the filtered gaseous phase (FGP), the smoke was introduced into the experimental vessels, from the syringe, via a 20-gauge needle attached to the stopcock. For other experiments, aqueous extracts of FGP were prepared by drawing either water or Hanks balanced salt solution (usually 1 ml/20 ml FGP) into the syringe containing the FGP and the whole agitated for about 2 min.

Rabbit alveolar macrophages. Alveolar macrophages were obtained from New Zealand White rabbits by washout of rabbit lungs, centrifuged and suspended in Hanks balanced salt solution according to the method of Green and Carolin.[3]

Histochemical "staining" of rabbit alveolar macrophages. Rabbit alveolar macrophages were incubated for 2 hr in a volume of 2 ml in plastic tissue culture flasks and the constitution of the incubation was as described by Green and Carolin.[3] Thus, control flasks contained macrophages ($2–3 \times 10^6$), autologous rabbit serum, specific immune rabbit serum and *Staphylococcus albus* suspension (approximately 10^5). Other flasks contained, in addition, either cysteine–HCl (5 μmoles) and/or 10 ml FGP. At the beginning and at the end of the incubation period, samples of the flask contents were used for the preparation of smears on microscope slides for subsequent histochemical investigation and samples were also taken for quantitative bacterial culture.[3] After 2 hr the flasks were drained and the cells which remained adhered to the flasks were also subjected to histochemical investigation.

Histochemical methods. Glucose 6-phosphate dehydrogenase activity was detected in macrophages by incubating the cells in a medium containing glucose 6-phosphate, TPN and neotetrazolium according to the method of Cohn and Way.[9] Enzyme activity was indicated by intracellular deposition of blue formazan crystals.

Histochemical demonstration of acid phosphatase activity was carried out by incubating the cells in a solution containing either α-naphthyl phosphate or naphthyl AS-phosphate according to the methods described by Barka and Anderson.[10] With α-naphthyl phosphate as substrate, reddish-brown precipitates marked the site of enzyme activity and with naphthyl AS-phosphate enzyme activity was indicated by the appearance of a vivid red azo dye in the cells.

Aldehyde dehydrogenase activity was indicated as blue deposits in macrophages following incubation in a reaction mixture containing NAD, nitro blue tetrazolium and benzaldehyde as substrate after the method described by Gabler.[11]

For the detection of glucose 6-phosphate dehydrogenase activity in macrophages, unfixed, air-dried smears were used. The smears were incubated in the appropriate media for 2 hr at 37°. Medium was also added to the drained incubation flasks which were similarly incubated. Smears were washed briefly with water and mounted in Ferrants medium. The cells in the flasks were assessed immediately for enzyme activity and no permanent preparations were made.

For the detection of acid phosphatase activity, air-dried smears were fixed in formol-calcium fixative, at 4°, usually for 3 hr, although longer fixation times were also used (up to 18 hr) with no apparent loss of phosphatase activity. After washing with water, cells were incubated at 37° for 30 min in the presence of the solutions containing either α-naphthyl phosphate or naphthyl AS-phosphate. Preparations were rinsed briefly with water and counterstained for 30–60 sec with methyl green at room temperature. Following rapid dehydration in alcohol and clearing in xylol, preparations were mounted in synthetic mounting medium.

Assay of crystalline glyceraldehyde 3-phosphate dehydrogenase. Crystals of the enzyme were dissolved in buffer or water to a concentration of 250 μg/ml, unless otherwise indicated, and used immediately. Substrate (glyceraldehyde 3-phosphate) was prepared from DL-glyceraldehyde 3-phosphate diethyl acetal barium salt by deionising on Dowex 50 ion-exchange resin and heating in boiling water for 3–5 min. The concentration of D-glyceraldehyde 3-phosphate was determined enzymically by measuring the reduction of NAD in the presence of glyceraldehyde 3-phosphate dehydrogenase. Enzyme activity assays were conducted after the method of Velick.[12] The assay mixture (3 ml) contained tris–HCl buffer (0·1 M, pH 8.5), NAD ($3{\cdot}3 \times 10^{-4}$ M), sodium arsenate (0·017 M), cysteine–HCl ($3{\cdot}3 \times 10^{-3}$ M adjusted to pH 7·0 immediately before use) and enzyme solution (0·1 ml). The reaction was initiated in the test cuvette by adding 0·1 ml of a solution containing 0·75 μmoles of glyceraldehyde 3-phosphate (0·1 ml of water was added to the control cuvette) and the increase in absorbancy was followed at 340 nm. The increase in absorbancy between 15 and 45 sec after addition of the substrate, was proportional to the enzyme concentration when the rate in increase in optical density was between 0·05 and 0·3/min[13] and enzyme solutions were thus diluted to suitable concentrations before assay. The results were expressed in enzyme units, a unit being defined as the amount of enzyme which produces an increase of optical density of 0·0001/min at 340 nm.[14]

Preparation of rabbit macrophage supernatants. The collection and preparation of alveolar macrophages by pulmonary lavage with Hanks balanced salt solution was as described by Green and Carolin.[3] The yield of cells from rabbits showed considerable variation ($14–140 \times 10^6$) and thus the number of macrophages used for each experiment was variable. However, cell incubates were prepared in which the number of macrophages in each incubation tube was approximately 10×10^6 cells. The total volume of each incubate was 8 ml and was 5 per cent with respect to autologous rabbit serum. In test incubates, additions of test materials were made proportionally with respect to the number of cells in the incubate and in control incubates the final volume (8 ml) was adjusted by the addition of Hanks balanced salt solution.

Incubation was for 2 hr at 37° after which the cells were harvested by centrifuging for 15 min at 1200 rev/min. The cells were then suspended in 5 ml of Hanks balanced salt solution at 37° and following centrifugation (1200 rev/min for 15 min) resuspended in 2 ml of sucrose (0·25 M) at 4°. Cell disintegration was achieved by sonication at 0° for 30–60 sec at intermediate intensity (Biosonik, Bronwill Scientific Co.) and when cell preparations had been examined microscopically for complete cell rupture, they were centrifuged at 18,000 *g* for 30 min at 0°. The supernatants were used for the determination of enzyme activity.

Determination of protein. The protein content of extracts of alveolar macrophages was determined by the method of Lowry, Rosebrough, Farr and Randall.[15]

Assay of glucose 6-phosphate dehydrogenase. Glucose 6-phosphate dehydrogenase activity in the supernatants was determined after the method of Glock and McLean[16] in the presence of excess glucose 6-phosphate and NADP. The reduction of NADP was followed spectrophotometrically at 340 nm in 1-cm cuvettes at room temperature. The reaction mixture consisted of 1·4 ml of tris–HCl buffer (0·08 M, pH 7·5), 0·5 ml of glucose 6-phosphate (0·01 M), 0·1 ml of NADP (0·0025 M), 0·5 ml of $MgCl_2$ (0·1 M) and cell supernatant (usually 0·2 ml). The final volume of the reaction mixture was adjusted to 3 ml with buffer. The reaction was initiated by the addition of substrate

solution to both test and reference cuvettes, the reference cuvette being devoid of NADP. (No reduction of NADP was recorded in the absence of substrate and in the reference cuvette NADP was replaced by water.)

The increase in optical density at 340 nm during the 5-min period, following the addition of substrate, was recorded and the average increase in OD/min over the 5-min period was calculated.[17] The results were expressed as μmoles NADPH produced/min/10^6 cells.

Assay of lactic dehydrogenase. Lactic dehydrogenase was assayed after the method of Reeves and Fimognari[18] by measuring the rate of decrease of absorbance of NADH at 340 nm in 1-cm cells. The assay system consisted of 1·9 ml of phosphate buffer (0·1 M, pH 7·4), 0·2 ml of sodium pyruvate (2·27 μmoles), 0·2 ml of bovine albumin (3 mg), 0·2 ml of NADH (0·5 μmoles) and cell supernatant (usually 0·2 ml). The final volume of the reaction mixture was adjusted to 3 ml with buffer and the reaction was initiated by the addition of substrate to both cuvettes, the reference cuvette being devoid of NADH (oxidation of NADH was not recorded in the absence of substrate and NADH was substituted by water in the reference cell). The decrease in optical density at 340 nm during the 2-min period following the addition of substrate, was recorded and the average decrease in OD/min over the 2-min period was calculated. The results were expressed as μmoles NADH oxidized/min/10^6 cells.

Assay of glyceraldehyde 3-phosphate dehydrogenase. Glyceraldehyde 3-phosphate dehydrogenase was assayed from both directions of the reaction. The assay mixture (total volume, 3 ml) for the forward reaction (glyceraldehyde 3-phosphate, G3-P → 3-phosphoglyceric acid, 3-PGA) contained Tris buffer (0·1 M, pH 8·5), NAD ($3{\cdot}3 \times 10^{-4}$ M), sodium arsenate ($1{\cdot}7 \times 10^{-2}$ M), cysteine–HCl ($3{\cdot}3 \times 10^{-3}$ M, adjusted to pH 7·0 immediately before use), G3-P (0·75 μmoles) and cell supernatant. The reaction was initiated by the addition of substrate to the test cuvette and an equal volume of water was added to the reference cuvette. (Reduction of NAD did not occur in the absence of substrate.) The optical density at 340 nm was recorded after 10 min and the results were expressed as μmoles NADH produced/10 min/10^6 cells.

The assay of the back reaction (3-PGA → G3-P) was coupled with 3-PGA kinase in a volume of 3 ml. The reaction mixture contained tris–HCl buffer (5×10^{-2} M, pH 7·6), NADH (9×10^{-4} M), $MgCl_2$ ($3{\cdot}5 \times 10^{-3}$ M), KCl ($2{\cdot}3 \times 10^{-2}$ M), 3-PGA ($1{\cdot}5 \times 10^{-2}$M), ATP ($4{\cdot}4 \times 10^{-3}$ M) and 3-PGA kinase (4 units) and cell supernatant. In the reference cuvette NADH was substituted by water. The decrease in optical density at 340 nm was recorded and, in each determination, the average decrease in OD/min was calculated. The results were expressed as μmoles NADH oxidized/min/10^6 cells. Enzyme activities of extracts of treated macrophages were expressed as percentages of values obtained with extracts of untreated cells. These percentages were virtually identical by the two methods of assay.

EXPERIMENTAL AND RESULTS

Histochemical studies

The results of experiments designed to detect aldehyde dehydrogenase activity were particularly striking, since it was shown that FGP inhibited enzyme activity. Thus, in 14 separate experiments (14 animals) out of a total of 16, formazan deposits could not be detected in cells treated with smoke. It was shown, however, that the cells

could be protected from loss of aldehyde dehydrogenase activity in the presence of cysteine. Thus, the degree of formazan deposition in macrophages which were incubated with FGP in the presence of cysteine was indistinguishable from that seen in cells in control flasks.

In addition, the results demonstrated that the loss of enzyme activity in macrophages treated with FGP coincided with loss of phagocytic competence. When quantitative bacteriological data revealed impairment of macrophage phagocytic function in the presence of FGP, aldehyde dehydrogenase activity could not be detected. In contrast, when cysteine was present, in addition to FGP, then enzyme activity together with phagocytic activity was restored. There existed, therefore, a complete correlation between the ability to phagocytose bacteria and the presence of active aldehyde dehydrogenase.

Response to the histochemical reaction for glucose 6-phosphate dehydrogenase was different in cells which had adhered to the flask surfaces than in those cells which were used for the preparation of smears. In 11 separate experiments, preparations of macrophages adhering to the flasks showed numerous groups of cells with heavy deposits of formazan crystals and it was not possible to distinguish between the staining intensity of cells in control flasks and those in flasks containing FGP. It would appear, therefore, that FGP does not inhibit the activity of glucose 6-phosphate dehydrogenase in macrophages which adhere to the surfaces of the flasks. This observation was also made with respect to cells of air-dried smears but in these cells a response to the presence of FGP was noted, since there was a greater intensity of staining in cells from flasks treated with FGP. In 15 out of a total of 18 separate experiments, there was a correlation between the degree of deposition of formazan crystals in the macrophages and the presence of FGP, the deposition being greater in the presence of FGP.

In all preparations of macrophages stained for acid phosphatase activity, the majority of the cells showed marked precipitation of the azo dye and no diminution in the intensity of the reaction in the presence of FGP was recorded.

Experiments with crystalline glyceraldehyde 3-phosphate dehydrogenase

Preliminary experiments were made in which enzyme (500 μg protein) was incubated with 8 ml FGP at 37° for periods of up to 2 hr in aqueous solution and in buffer (tris–HCl, 4×10^{-2} M) at pH 7·0, 7·5 and 7·7. Marked decreases in enzyme activity (up to 75 per cent) were recorded under all conditions. It was also shown that the presence of FGP did not result in pH changes in aqueous solutions of the enzyme and in all subsequent experiments aqueous solutions of the enzyme were used.

During preliminary experiments, it became apparent that although marked decreases of enzyme activity were always recorded in the presence of 8 ml FGP, the extent of inhibition was variable. It was possible to correlate this variability with the "staleness" of the cigarettes since, in general, greater inhibitions were recorded when cigarettes from newly-opened packets were used.

Effect of incubation time on the inhibition of enzyme activity in the presence of 8 ml FGP. Experiments were made at two concentrations of enzyme; 1000 μg protein/2 ml and 500 μg protein/2 ml. Incubations at 37° were in tissue culture flasks; each flask containing 2 ml of enzyme solution and 8 ml of FGP. Control flasks which did not contain FGP were assayed at zero time and after 2 hr. There was no significant

difference in the activity of the controls over the 2-hr period. Test flask contents were assayed at various times over 2 hr. At the higher enzyme concentration, activity was inhibited by approximately 50 per cent after 5 min, while after 2 hr only 20 per cent of the activity remained. Substantially the same results were recorded at the lower concentration in that the enzyme was markedly inhibited during the first 5 min and the extent of inhibition increased slowly over 2 hr (Fig. 1). The degree of inhibition was, however, greater; approximately 60 per cent inhibition in 5 min and 90 per cent inhibition in 2 hr.

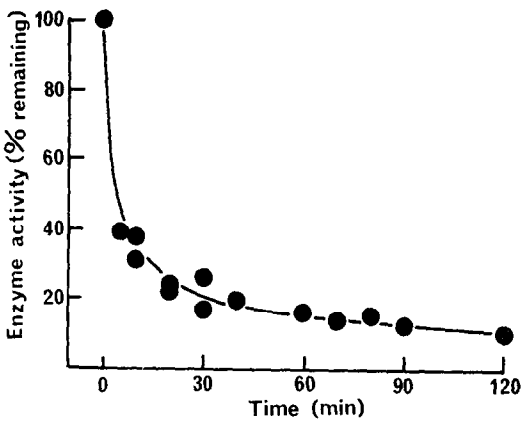

FIG. 1. Effect of incubation time on the activity of glyceraldehyde 3-phosphate dehydrogenase in the presence of 8 ml of FGP. For details see text.

Effect of concentration of FGP on enzyme activity. Flasks, each containing 2 ml of enzyme solution (500 μg protein) were incubated at 37° for 2 hr in the presence of FGP (2–24 ml). Control flasks, not containing FGP, were assayed after 2 hr at 37°. The results are recorded in Fig. 2. With 2 ml FGP, approximately 43 per cent of the enzyme activity remained and there was a gradual decrease in activity with increasing

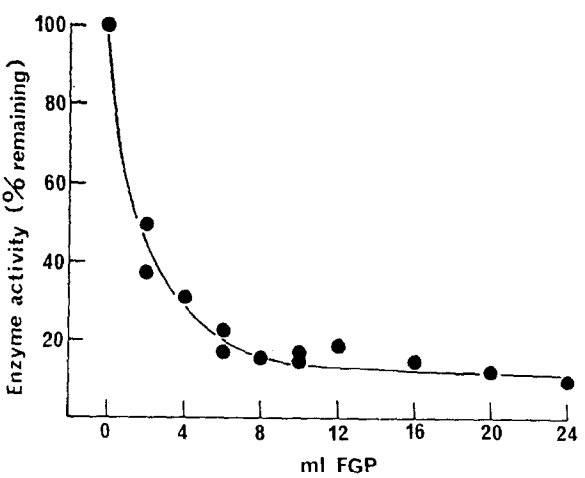

FIG. 2. Effect of the concentration of FGP on the activity of glyceraldehyde 3-phosphate dehydrogenase. For details see text.

amounts of smoke up to 8 ml. Therefore, increased concentrations of FGP did not substantially affect the percentage inhibition.

Effect of 8 ml FGP on various concentrations of enzyme. Flasks, each containing 2 ml of enzyme solution (200–2000 μg protein) were incubated for 2 hr in the presence of 8 ml of FGP. Control flasks without FGP were also incubated and assayed after 2 hr. The results are recorded in Fig. 3 and show that the percentage inhibition of the enzyme is related to the relative concentration of FGP with respect to the enzyme.

Effect of aqueous FGP on the activity of the enzyme and the effect of the presence of cysteine. The effect of aqueous extracts of FGP was measured at time intervals ranging from 10 min to 2 hr at 37°. The aqueous solution of FGP was added to the incubation mixture so that the concentration was equivalent to 8 ml gaseous FGP/500 μg of enzyme. Other incubation mixtures contained aqueous FGP and enzyme together with solutions of cysteine-HCl (2·5 μmoles/8 ml FGP) which were adjusted to pH 7·0 immediately before use. Aliquots of the reaction mixtures were removed, at the times shown in Fig. 4, and assayed for enzyme activity.

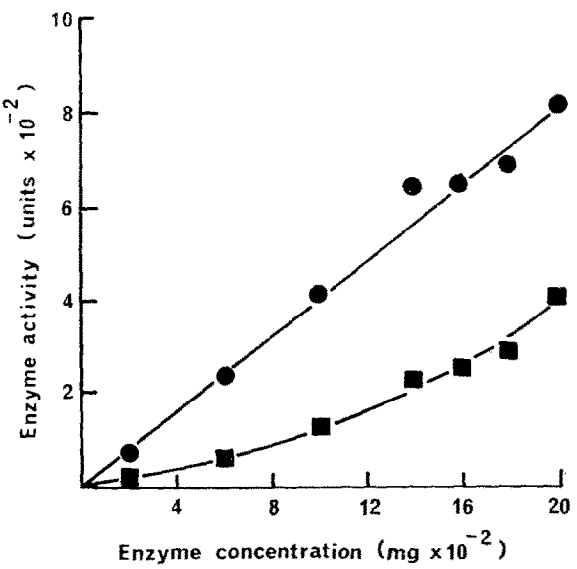

FIG. 3. Effect of 8 ml of FGP on glyceraldehyde 3-phosphate dehydrogenase. Various concentrations of enzyme were incubated at 37° for 2 hr in the absence (●—●) and presence (■—■) of smoke. For details see text.

Inhibition of enzyme activity by aqueous FGP was almost identical with that recorded with gaseous FGP (see Fig. 1). The results also show that the enzyme is not inhibited by FGP in the presence of cysteine. Other experiments designed to test the protective effect of cysteine in the presence of gaseous FGP confirmed this finding.

Experiments were also made in which FGP and enzyme (8 ml FGP/500 μg protein) were incubated for 2 hr at 37° prior to the addition of cysteine-HCl (2·5 μmoles/8 ml FGP). The enzyme was assayed before the addition of cysteine and after further incubation (for 1 hr) in the presence of cysteine. After the first incubation period,

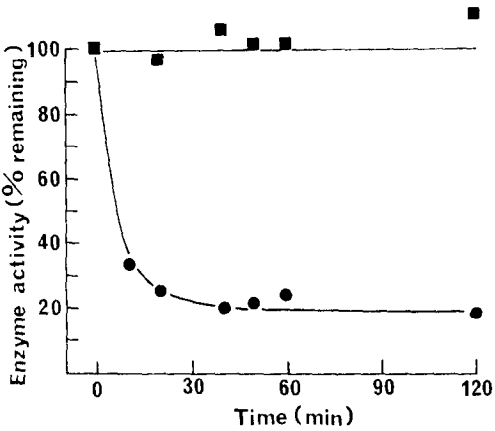

FIG. 4. Effect of aqueous FGP on the activity of glyceraldehyde 3-phosphate dehydrogenase in the presence (■—■) and absence (●—●) of cysteine–HCl. For details see text.

approximately 15 per cent of the activity remained and no significant increase in activity was recorded in the second incubation period in the presence of cysteine.

Determination of dehydrogenase activity in alveolar macrophages

Preliminary experiments were made to determine the reproducibility of the method of preparation of macrophage supernatants. Separate cell extracts were prepared from three rabbits, and the supernatants were assayed for glucose 6-phosphate dehydrogenase activity and protein content. The results (Table 1) show that the preparations were reproducible, within narrow limits, with respect to both protein content and glucose 6-phosphate dehydrogenase activity.

TABLE 1. PROTEIN CONCENTRATION AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6PD) ACTIVITY OF RABBIT ALVEOLAR MACROPHAGE PREPARATIONS

Animal	Total number of cells obtained	Protein (μg/10^6 cells)	G6PD activity (nmoles TPNH formed /min/10^6 cells)	G6PD activity (nmoles TPNH formed /μg protein)
1	35 × 10^6	105	15·0	0·143
2	24·4 × 10^6	113	16·6	0·147
3	26 × 10^6	93	14·5	0·155

Assay of enzyme activity in macrophages treated with FGP. Dehydrogenase activity was measured in control incubates (containing cells in Hanks solution) and test incubates (containing cells together with FGP and/or cysteine–HCl). FGP (4 ml/10^6 cells) was dissolved in Hanks solution (usually 1 ml/20 ml FGP) and added to the incubation medium. In incubates containing cysteine, the amount of cysteine was proportional to the FGP concentration (2 mg/10 ml FGP).

Glucose 6-phosphate dehydrogenase activity, in the presence and absence of FGP, was measured in cell extracts obtained in 11 separate experiments. The absolute values of enzyme activity did not show significant variation from one cell preparation to another, and further, no significant difference in activity was recorded in the presence of FGP or FGP together with cysteine (four experiments).

Similar findings were recorded with respect to lactic dehydrogenase activity which was measured in nine experiments in the presence and absence of FGP. Enzyme activity did not show significant variation from one control preparation to another and no significant variation of activity was recorded in the presence of FGP or in the presence of FGP and cysteine.

Markedly different results were recorded when glyceraldehyde 3-phosphate dehydrogenase activity was measured (see Fig. 5). The activity of this enzyme was adversely

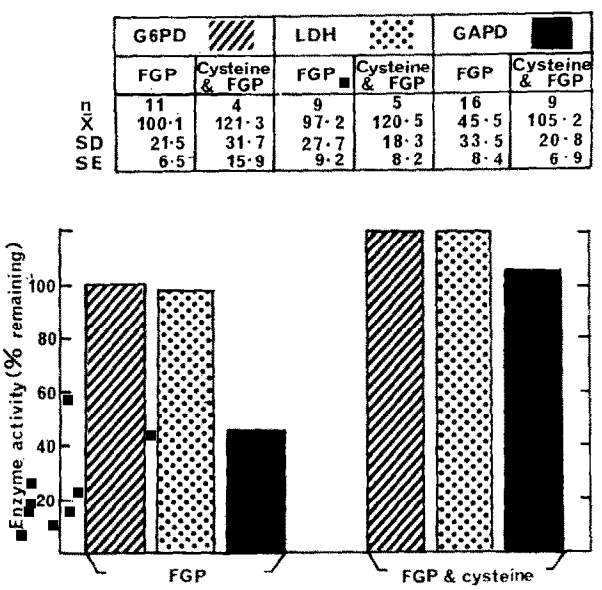

FIG. 5. Effect of FGP, in the absence and presence of cysteine, on the activities of glyceraldehyde 3-phosphate dehydrogenase (GAPD, ■), glucose 6-phosphate dehydrogenase (G6PD, ▨), and lactic dehydrogenase (LDH, ▩) of rabbit alveolar macrophages treated with aqueous extracts of FGP. The number of experiments is indicated and statistical data is given in the inset. For details see text.

affected by the presence of FGP. Enzyme activity in cells of control incubates showed considerable variation but the extent of the effect of FGP could not be correlated with the level of enzyme activity in control cells. In addition, the degree of inhibition of enzyme activity by FGP varied from one experiment to another. Thus, in a total of 16 experiments, 72–93 per cent inhibition was recorded in eight experiments, 26–55 per cent inhibition in five experiments, while in three experiments virtually no inhibition was recorded. In experiments in which cells were treated with FGP in the presence of cysteine, no significant inhibition of enzyme activity was detected.

Separate experiments were made to determine the effect of the concentration of FGP on the activity of macrophage glyceraldehyde 3-phosphate dehydrogenase. Enzyme

TABLE 2. EFFECT OF AQUEOUS EXTRACTS OF FGP, AT TWO CONCENTRATIONS (2 ml FGP/10^6 CELLS AND 4 ml FGP/10^6 CELLS) ON THE ACTIVITY OF GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE ACTIVITY IN RABBIT ALVEOLAR MACROPHAGES

Experiment	Enzyme activity* in the presence of	
	2 ml FGP/10^6 cells	4 ml FGP/10^6 cells
1	42·0	5·0
2	63·3	22·0
3	58·0	20·8

* Values are given as percentages of control determinations made in the absence of FGP.

activity was measured in cells treated with 2 ml FGP/10^6 cells and 4 ml FGP/10^6 cells. The results are recorded in Table 2 and show, as previously noted, a variation in the percentage inhibition of the enzyme from one experiment to another. However, the figures demonstrate that the extent of inhibition is markedly affected by the concentration of FGP.

DISCUSSION

In summary, the results of experiments reported here demonstrate the potency of FGP and aqueous extracts of FGP as inhibitors of glyceraldehyde 3-phosphate dehydrogenase activity. This was shown by investigating the inhibition of purified preparations of the enzyme under a variety of conditions. It has also been shown, both histochemically and quantitatively, that the enzyme is severely inhibited in macrophages treated with FGP. When cysteine was present in addition to FGP no inhibition of enzyme activity was recorded either *in vitro* or *in vivo*. The study further revealed a close correlation between loss of phagocytic competence by alveolar macrophages, on the one hand, and reduction of glyceraldehyde 3-phosphate dehydrogenase activity on the other.

The metabolic concomitants of phagocytosis and subsequent degranulation have been investigated by Oren, Farnham, Saito, Milofsky and Karnovsky[8] and with respect to polymorphonuclear leukocytes, it is established that operation of the glycolytic pathway is necessary for particle uptake while the activity of the hexose monophosphate shunt is a prerequisite for the bactericidal effect (see also Holmes, Page and Good[19]). The situation with respect to alveolar macrophages is less clear, but, nevertheless, it has been established that glucose metabolism via glycolysis and the hexose monophosphate pathway is increased during phagocytosis[20] and that inhibition of the glycolytic pathway severely impairs particle uptake.[8] In this context, the results obtained when rabbit alveolar macrophages, treated with FGP, were "stained" for glucose 6-phosphate dehydrogenase and aldehyde dehydrogenase assume considerable significance. While glucose 6-phosphate dehydrogenase accomplishes only one transformation in the hexose monophosphate pathway, it occupies a key

position in glucose metabolism in the cell[21] and other investigators[9,22] have employed histochemical determination of glucose 6-phosphate dehydrogenase to assess the activity of the hexose monophosphate pathway. The observation reported here that glucose 6-phosphate dehydrogenase is not inhibited by FGP strongly suggests that the metabolic conversion catalysed by this enzyme is unaffected by the constituents of filtered smoke. Further, the results obtained with air-dried smears of cells treated with FGP indicate that the activity of the enzyme may be increased.

The findings of the study of aldehyde dehydrogenase activity are particularly pertinent in the light of the observation that inhibition of glycolysis impairs macrophage phagocytic activity. The results of histochemical detection presented here provide evidence for the inhibition of the enzyme by FGP and protection from the effects of FGP by cysteine. Moreover, these effects are paralleled by the phagocytic data: when the aldehyde dehydrogenase activity is inhibited, phagocytic activity is depressed and when inhibition is prevented by protection with cysteine, phagocytic ability is indistinguishable from that of untreated cells. By extrapolation of these findings, it may be suggested that, in the presence of FGP, the glycolytic pathway is impaired while the first reaction of the hexose monophosphate pathway is unaffected.

Experiments involving crystalline glyceraldehyde 3-phosphate dehydrogenase showed that FGP and aqueous extracts of FGP are potent inhibitors of enzyme activity. Further, the degree of inhibition is dependent upon the length of the preincubation period and the amount of enzyme relative to the FGP concentration. In the presence of cysteine, FGP no longer acts as an inhibitor of the enzyme. Involvement of thiol groups in the enzymic reaction is well known and it has been established that the sulphur must be maintained in the reduced form for maximal activity and that the enzyme is stabilized by cysteine.[23] Consideration of this data allows some interpretation of the effect of cigarette smoke on the dehydrogenase and it is interesting to compare the inhibition of the enzyme by FGP with inhibition by iodoacetate studied by other workers. Webb[24] has emphasized that very few SH enzymes react completely with measurable concentrations of iodoacetate within 30 min and App and Meiss[25] have shown that the inhibition of alcohol dehydrogenase by iodoacetate (1 mM) does not reach a maximum after 120 min. Similarly, the inhibition of glyceraldehyde 3-phosphate dehydrogenase by FGP is by no means instantaneous and a 2-hr preincubation period was required in some experiments before the full inhibitory effect was realized. Cori, Slein and Cori[26] noted that inhibition of the enzyme by iodoacetate was not immediate and that the inhibition was not reversed by subsequent treatment with cysteine. Similarly, in the present study, although glyceraldehyde 3-phosphate dehydrogenase is protected from the inhibitory effects of FGP by cysteine, enzyme activity lost by FGP treatment is not restored by subsequent treatment with cysteine. Webb[24] has recorded the view that protection experiments in which a thiol is added previous to, or with the SH reagent, are of little value since they merely demonstrate the effect of reducing the concentrations of the free SH reagent, assuming that it reacts with the thiol. He does, however, point out that such experiments are useful in the context of practical determination of the ability of substances to reduce the toxic effects of SH reagents. In the present study, the experiments demonstrating the protective effect of cysteine provide important information for this purpose and, perhaps more importantly, provide a strong indication that FGP acts as a sulphydryl reagent.

The potency of FGP as an inhibitor of glyceraldehyde 3-phosphate dehydrogenase *in vivo* was confirmed in experiments in which enzyme activity was measured in macrophages treated with aqueous FGP. It was shown that while this dehydrogenase is inhibited, the activities of glucose 6-phosphate dehydrogenase and lactic dehydrogenase in the same cells are not significantly altered. At the present time it is not possible to explain the small number of experiments in which glyceraldehyde 3-phosphate dehydrogenase was not affected by the presence of FGP, but it is noteworthy that the loss of competence with respect to phagocytosis by alveolar macrophages is not invariable and, moreover, the extent of inhibition of phagocytosis by FGP is variable (Green, unpublished results). This latter observation is particularly interesting when regarded in the context of the present findings which show that the extent of inhibition of glyceraldehyde 3-phosphate dehydrogenase is also variable. It is particularly interesting to note that glyceraldehyde 3-phosphate dehydrogenase activity is not inhibited in macrophages treated with FGP in the presence of cysteine and the enzyme levels are not distinguishable from those of control cells. A parallel situation exists with respect to phagocytosis in the presence of FGP and cysteine since, under such conditions, the cells are protected from the effects of FGP and phagocytosis proceeds normally.[4]

Green and Carolin[3] reported data which showed a correlation between FGP concentration and the extent of loss of alveolar macrophage phagocytic ability. In the present study, a correlation between FGP concentration and the extent of inhibition of glyceraldehyde 3-phosphate dehydrogenase was shown. Thus, it again seems possible to correlate the phagocytic and enzymological phenomena.

Collectively, these findings point to a relationship between macrophage phagocytic ability and the activity of glyceraldehyde 3-phosphate dehydrogenase since loss of phagocytic competence and loss of enzyme activity appear to be concomitant. While it is extremely unlikely that the inhibition of glyceraldehyde 3-phosphate dehydrogenase is the only biochemical defect resulting from exposure to cigarette smoke, the results discussed here make it possible to postulate, with some confidence, that the reduction of glyceraldehyde 3-phosphate dehydrogenase activity is intimately related to reduction of phagocytic competence. It is further suggested that inactivation of the dehydrogenase leads to impairment of the glycolytic pathway and the consequent disturbance of energy metabolism may be responsible for the observed functional defect in macrophages treated with smoke.

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