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PATSY WANG, RICHARD C. FRANSON, LAWRENCE R. DECHATELET, MOSELEY WAITE, AND CHARLES E. MCCALL

The Departments of Biochemistry and Medicine, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103

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The effect of methylprednisolone sodium succinate treatment on Bacillus Calmette-Guérin (BCG) immunization was examined in alveolar macrophages of New Zealand White rabbits. Intravenous BCG injection caused a marked increase in cell number and in the hexosemonophosphate shunt activity. There was also a selective augmentation in the activity of acid phosphatase and β-glucuronidase and an increase in lysosomal density. Activity of the lysosomal phospholipases was decreased. Steroid treatment suppressed the BCG-induced increase in cell number, hexosemonophosphate shunt activity, acid phosphatase, and β-glucuronidase, and prevented the increase in lysosomal density. On the other hand, steroid treatment increased the activity of the phospholipases to levels higher than either control values or those obtained with cells from BCG-treated rabbits.

Cohn and Wiener (7) demonstrated the separation of lysosomes from mitochondria obtained from alveolar macrophages of Bacillus Calmette-Guérin (BCG)-induced rabbits by isopycnic sucrose gradient centrifugation. Franson and Waite (*manuscript in preparation*) compared the lysosomes of alveolar macrophages obtained from control and from BCG-stimulated animals by isopycnic sucrose gradient centrifugation and found that the former sedimented with the mitochondria, whereas the latter sedimented in a denser fraction similar to that reported by Cohn and Wiener (7). Myrvik et al. (15) earlier reported certain changes in the alveolar macrophage metabolism mediated by BCG immunization. They noted increases in the activities of certain lysosomal enzymes: acid phosphatase, β-glucuronidase, and lysozyme, as well as a significant elevation in the hexose monophosphate shunt activity, as measured by glucose-*1*-^{14}C oxidation. Subsequently, McCall et al. (3) demonstrated that the administration of methylprednisolone sodium succinate suppressed the BCG-mediated increase in glucose-*1*-^{14}C oxidation.

This study was undertaken to determine the effects of methylprednisolone on other BCG-mediated changes in the alveolar macrophage, the increase in lysosomal density, and the increases in the enzymatic activities of acid phosphatase and β-glucuronidase. The present communication extends previous work in demonstrating that methylprednisolone will likewise suppress the BCG-induced changes in lysosomal density and the activity of some, but not all, lysosomal enzymes.

MATERIALS AND METHODS

Materials. Glucose-*1*-^{14}C (specific activity, 52.2 mCi) was obtained from the New England Nuclear Corp., Boston, Mass. 1-Acyl-2-[^{14}C]-linoleoyl-3-glycerophosphorylethanolamine was synthesized by the procedure of Waite and van Deenen (21). Hydroxide of hyamine came from Packard Instrument Co., Downers Grove, Ill. Tetracycline, obtained from Rachelle Lab., Inc., Long Beach, Calif., as Tetrachel-Vet Bulk-25, a soluble powder (25 g of tetracycline hydrochloride per lb), was administered in the drinking water at a concentration of 1.2 g/gal (1.2 g/3.8 liters). Methylprednisolone sodium succinate (Solu-Medrol) was the generous gift of the Upjohn Co., Kalamazoo, Mich. Heat-killed BCG was kindly donated by Quentin Myrvik. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

Vaccination, steroid administration, and cell collection. A sonic suspension of a heat-killed BCG in Bayol F (100 μg in 0.1 ml) was injected into the marginal ear veins of female New Zealand White rabbits (1.5–2.0 kg). Intramuscular administration of methylprednisolone sodium succinate (Solu-Medrol) was begun concurrently with BCG injection. One group of rabbits received Solu-Medrol alone (3.5 mg/kg); two other groups were first injected with BCG and then received injections of Solu-Medrol (3.5 mg/kg and 30.0 mg/kg, respectively). The lower dose of steroid was administered on a daily basis, while the higher dose was given every other day for 3.5 weeks. A control group received no treatment, while another group was injected only with BCG.

Animals were sacrificed by air embolism, and the

alveolar macrophages were collected by the lung lavage technique described by Myrvik et al. (14). Lungs (8–10 g wet weight) from control and steroid-treated animals yielded 0.1 to 0.3 ml of packed cells, approximately 3.5×10^7 to 1×10^8 cells. Treatment with BCG caused an increase in lung weight (15–25 g wet weight), which was accompanied by a notable granulomatous response as well as an increase in volume (1–2.5 ml packed cells, equivalent to 3.5×10^8 to 8×10^8 macrophages). Lungs obtained from animals treated with both BCG and steroid were comparable to the lungs of control (8–10 g wet weight), but yielded 0.5 to 0.7 ml of packed cells per lung in weight. Granulomata were observed, but they were markedly smaller and less confluent than in the BCG-induced animals. Animals showing gross evidence of intercurrent infection, as characterized by abnormal lungs and cell volume, were not used in the study.

Lungs were lavaged with 80 ml of isotonic saline, followed by two washes with the same medium. Contaminating red blood cells were lysed for 20 sec with deionized water, and the suspension was brought to isotonicity with 3.5% saline. The suspension was centrifuged, and the pellet was suspended in 0.25 M sucrose in a ratio of 1 ml of packed cells to 8 ml of 0.25 M sucrose. Homogenization (30 strokes) was carried out with a tight-fitting Dounce homogenizer. Nuclei, cell debris, and the remaining intact cells were sedimented by centrifugation of the homogenate at $1,000 \times g$ for 15 min. Four milliliters of the supernatant solution were layered above a linear sucrose gradient (0.73 M to 1.61 M in 24 ml), which was cushioned with 6 ml of 1.93 M sucrose. The samples were placed in a Beckman SW25.1 swinging bucket rotor and centrifuged at 24,000 rev/min ($64,000 \times g$) for 2 hr in a Spinco model L ultracentrifuge. At the end of the isopycnic sucrose gradient centrifugation, the bottom of the centrifuge tube was punctured, and fractions were collected from the top of the gradient by pumping 1.93 M sucrose from below. The fraction layered over the gradient (4 ml) was pumped off, and thereafter nine fractions of 3.0 ml each were collected. The density of the gradient fractions was measured for each experiment with a Bausch and Lomb refractometer. Cytochrome oxidase and reduced triphosphopyridine nucleotide (TPNH) cytochrome *c* reductase assays were performed on samples as they came from the gradient. For the other enzyme assays, the samples first were dialyzed for 12 hr against 8 liters of 1 mM sodium acetate (*p*H 5.0) to rupture the lysosomes and to remove sucrose. Assays including Triton X-100 revealed no latent enzymatic activity.

Enzyme assays. Hexosemonophosphate shunt activity (HMS), as estimated by the evolution of $^{14}CO_2$ from glucose-*1*-^{14}C, was examined in intact cells by a modification of a method previously described (8). A comparison was made of activities in resting cells and in cells stimulated by the addition of 0.15 ml of latex particles (0.8 μm in diameter). Each incubation flask contained 1.55 ml of phosphate-buffered saline (PBS), 0.10 ml of glucose-*1*-^{14}C (0.20 μCi), 0.20 ml of 80 mg% D-glucose, and 0.15 ml of PBS or latex (at a ratio of 100 particles per cell). The reaction was initiated by the addition of 5×10^6 cells in a volume of 1.0 ml and terminated with 1 ml of 5% trichloroacetic acid after incubation at 37 C for 1 hr. $^{14}CO_2$ released during the course of the incubation was trapped in hydroxide of hyamine and counted in a Packard scintillation counter.

Phospholipase A was assayed by the procedure of Waite and van Deenen (21). Phospholipases A_1 and A_2 (E.C. 3.1.1.4, lysosomal), respectively, hydrolyze the fatty acid ester linkages in the C-1 and C-2 positions of phospholipids to yield the monoacyl analogues and free fatty acids. Reaction mixtures in a total volume of 1.0 ml contained 75 nmoles of 1-acyl-2-[^{14}C]-linoleoyl-3-glycerophosphorylethanolamine (12,000 counts per min added as an aqueous ultrasonic suspension), 2 μmoles of ethylenediaminetetracetic acid (EDTA), 100 μmoles of sodium acetate buffer (*p*H 4.0), and 25 to 100 μg of protein. Reaction mixtures were incubated at 37 C for 1 hr, and the reaction was terminated by the addition of two volumes of methanol. The products were extracted by the method of Bligh and Dyer (4), and the radioactive lipids were separated by thin-layer chromatography on Silica Gel G plates by successive development in chloroform-petroleum ether (boiling point, 53–75 C)-acetic acid (70:32:2, v/v/v) and in chloroform-methanol-H_2O (70:30:4, v/v/v). The lipids were visualized with I_2 vapor, and the silicic acid containing the radioactive products was counted in a Nuclear-Chicago Corp. scintillation counter.

Cytochrome oxidase (E.C. 1.9.3.1, mitochondrial) and TPNH cytochrome *c* reductase (E.C. 1.6.99.1, microsomal) activities were determined by a modification of the method of Sottocasa et al. (18). Cytochrome *c* (type VI) was reduced by the addition of sodium borohydride, and the solution was neutralized with 0.5 N hydrochloric acid. β-Glycerophosphate was used to determine the activity of acid phosphatase (E.C. 3.1.3.2, lysosomal), as described by Gianetto and deDuve (10). Inorganic phosphate was measured by the turbidimetric procedure of Eibl and Lands (9). β-Glucuronidase activity (E.C. 3.2.1.3, lysosomal) was measured by Canonico's and Bird's modification (5) of the method of Talalay et al. (19). Protein was determined by the method of Lowry et al. (12), by using bovine serum albumin as a standard.

RESULTS

Table 1 shows the HMS activities of alveolar macrophages from control and treated animals. Phagocytosis in normal cells caused a 1.8-fold increase in $^{14}CO_2$ evolution over resting values. Steroid treatment alone did not alter the oxidation of glucose-*1*-^{14}C significantly. Although the resting and phagocytizing values for steroid-treated animals are slightly higher than the normal control values, the phagocytizing-to-resting (P-R) ratio of 1.7 is comparable to the normal control value. Cells from BCG-induced animals exhibited a marked increase in both resting and phagocytizing states, with an overall P-R ratio of 4.0. The BCG-steroid-treated animals yielded a P-R ratio that was lower than the control at

both doses studied. It was not possible to compare absolute values from experiment to experiment due to the biological variations. The tabulated values in these experiments, as well as those in Table 2, are taken from one study, which is representative of the trends observed in three different experiments.

The protein content and the specific activities of acid phosphatase, β-glucuronidase, and phospholipases A_1 and A_2, and the cytochrome oxidase of homogenates are collated in Table 2. There was an increase in the specific activities of acid phosphatase (2.2-fold) and β-glucuronidase (1.5-fold). In contrast to the increase in activity of acid phosphatase and β-glucuronidase, the specific activities for the two phospholipases and cytochrome oxidase were lower in the BCG-treated animals than in the control animals. Steroid treatment alone did not alter the activities of acid phosphatase or β-glucuronidase, but it caused an increase in the specific activity of both phospholipases. The enzymatic activities from BCG-steroid-treated alveolar macrophages for both doses of steroid were comparable to control values. Biological variations made it difficult to compare the absolute values obtained from different experiments.

The distributions of acid phosphatase, β-glucuronidase, phospholipases A_1 and A_2, cytochrome oxidase, and TPNH cytochrome *c* reductase are presented in Fig. 1 to 5 for the different conditions studied. For the control, steroid, and BCG-steroid-treated animals, the lysosomal

TABLE 1. *Oxidation of glucose-1-^{14}C by resting and phagocytizing alveolar macrophages from rabbits treated with steroid and BCG*

Condition	Counts/min in $^{14}CO_2$		
	Resting	Phagocytizing[a]	P-R[b]
Control	1,030 (1,008–1,040)	1,836 (1,817–1,854)	1.8
Steroid	1,252 (1,134–1,368)	2,096 (1,975–2,296)	1.7
BCG	2,697 (2,466–2,927)	10,808 (10,343–11,274)	4.0
BCG-steroid 3.6 mg/kg	896 (971–929)	1,039 (1,012–1,056)	1.2
30.0 mg/kg	1,074 (1,013–1,136)	1,344 (1,338–1,352)	1.2

[a] Latex particles were added to the incubation mixture in a ratio of 100 particles per cell.

[b] Ratio of phagocytizing to resting values. For the normal, steroid, and the BCG-steroid conditions, five to six animals were pooled for each experiment. Values were obtained from individual animals in the BCG study. The values in parentheses represent the range of triplicate determinations.

TABLE 2. *Protein content and specific activities in homogenates of alveolar macrophages from rabbits treated with steroid and BCG*

Determinations	Specific activity/mg of protein[a]			
	Control (4.2 mg of protein/ml)	Steroid (5.2 mg of protein/ml)	BCG-induced (7.5 mg of protein/ml)	BCG-steroid (4.1 mg of protein/ml)
Acid phosphatase	37	36	83	44
β-glucuronidase	69	78	105	74
Phospholipase A_1	60	99	31	76
Phospholipase A_2	83	156	46	104
Cytochrome oxidase	21		9	25

[a] Nanomoles of product formed per minute (for β-glucuronidase, nanomoles of product formed per hour). For the normal, steroid, and the BCG-steroid conditions, five to six animals were pooled for each experiment. Values were obtained from individual animals in the BCG study.

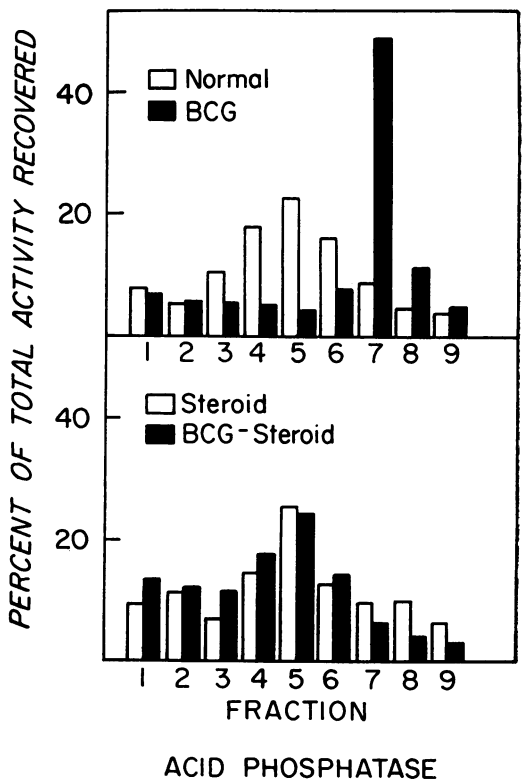

FIG. 1. *Distribution of acid phosphatase after sucrose gradient centrifugation. Enzymatic activity is expressed on the ordinate as the percent of the total activity recovered from the gradient.*

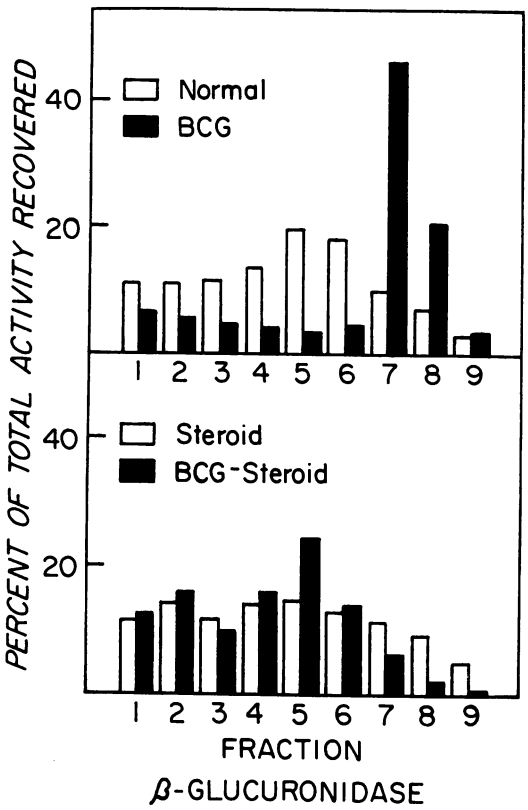

FIG. 2. *Distribution of β-glucuronidase after sucrose gradient centrifugation. Enzymatic activity is expressed on the ordinate as the percent of the total activity recovered from the gradient.*

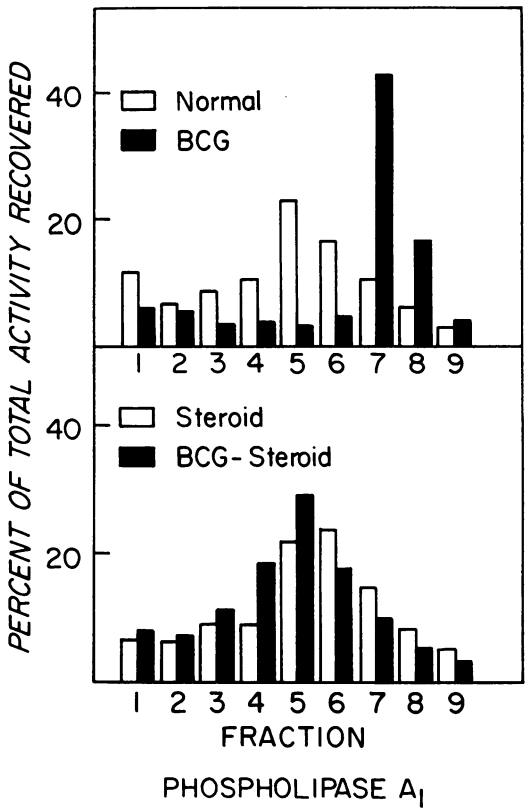

FIG. 3. *Distribution of phospholipase A_1 after sucrose gradient centrifugation. Enzymatic activity is expressed on the ordinate as the percent of the total activity recovered from the gradient.*

marker enzymes (acid phosphatase and β-glucuronidase) distributed in the same pattern as cytochrome oxidase, a mitochondrial marker enzyme. Most of the activity was found in fractions 4 to 6 (average sucrose density, 1.18). Treatment with BCG increased the lysosomal density, and the lysosomal marker enzymes were recovered primarily in fractions 7 and 8 (average sucrose density, 1.23), whereas the position of cytochrome oxidase in the gradient did not change. This BCG-induced shift was completely suppressed in the BCG-steroid-treated animals at both levels of steroid used. Fig. 1 and 2 illustrate the distribution patterns of acid phosphatase and β-glucuronidase under the four conditions studied. Lysozyme, also a marker enzyme for lysosomes, distributed in the same manner as did acid phosphatase and β-glucuronidase under all four conditions (data not shown). Profiles for phospholipases A_1 and A_2 are presented in Fig. 3 and 4. The similar shift in distribution of activity upon stimulation by BCG led Franson and Waite (*manuscript in preparation*) to conclude that the phospholipases A_1 and A_2 found in the alveolar macrophage which are active at *p*H 4.0 are of lysosomal origin.

Fig. 5 illustrates the distribution of marker enzymes for mitochondria (cytochrome oxidase) and microsomes (TPNH cytochrome *c* reductase). The data presented are for the BCG-steroid-treated animals, but no differences were detected between these and the normal, BCG-induced, and steroid-treated animals. Since the distribution of both cytochrome oxidase and TPNH cytochrome *c* reductase was similar for the four conditions studied, it appears that the effect of BCG and steroid treatment on the densities of the cell organelles examined is specifically manifested in the lysosomes.

DISCUSSION

In conjunction with the proliferation of the alveolar macrophages and the migration of the circulating monocytes to the pulmonary alveoli

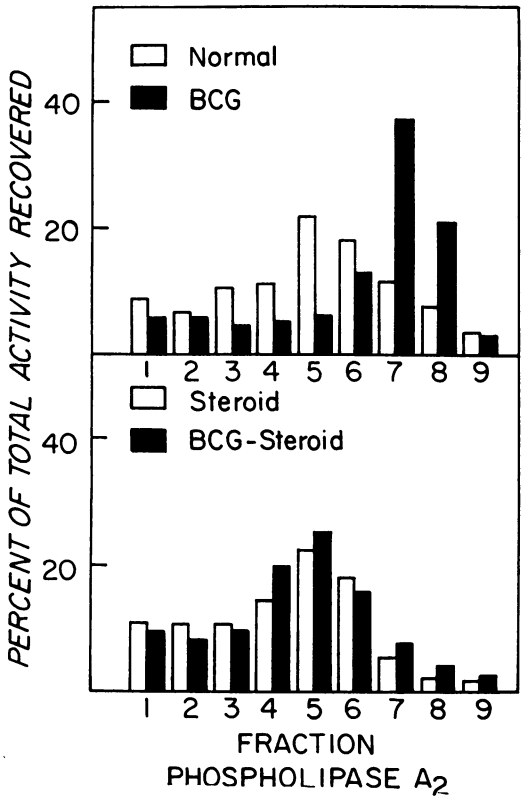

FIG. 4. *Distribution of phospholipase A_2 after sucrose gradient centrifugation. Enzymatic activity is expressed on the ordinate as the percent of the total activity recovered from the gradient.*

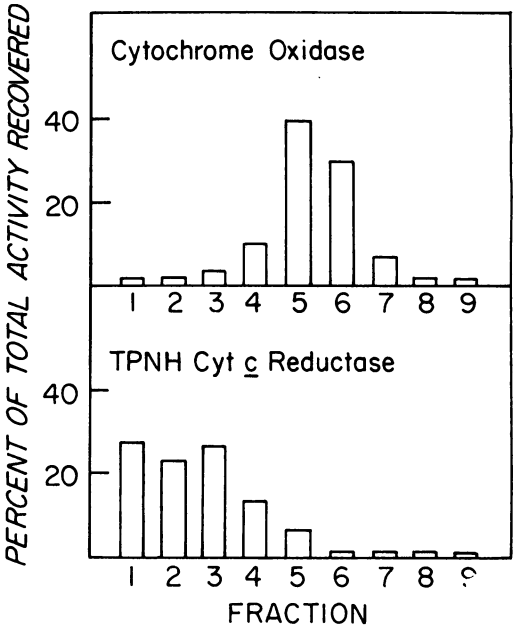

FIG. 5. *Distributions of cytochrome oxidase and TPNH cytochrome c reductase after sucrose gradient centrifugation. The data presented are for the BCG-steroid-treated animals, but no differences were detected between these and the normal, BCG, and steroid treated. Enzymatic activity was expressed on the ordinate as the percentage of the total activity recovered from the gradient.*

in the immunologic response to BCG injection, there is an activation process whereby the lysosomes within the macrophages increase in number and change in morphology (11, 17). Vaccination with BCG increases the activity of many, but not all, acid hydrolases contained within these granules. Associated with this activation process is a selective increase in density of the lysosomes compared with rat liver lysosomes (2, 3, 7) and with normal macrophage lysosomes (Franson and Waite, *manuscript in preparation*). As shown in this study, steroid treatment of BCG-vaccinated animals also seems to exert a selective effect; it suppresses the BCG-induced increase in lysosomal density without altering the mitochondrial or microsomal density. In connection with the suppression of the density increase, steroid also inhibits the increase in specific activities of only certain lysosomal enzymes of BCG-vaccinated animals. Although at this time the basis of this specificity is not known, some possibilities exist. The lysosomal enzymes may not be equally distributed among the lysosomes, and the BCG or steroid treatment may exert selective effects on different lysosomes. On the other hand, it is conceivable that the treatments affect the synthesis of the enzymes to different extents.

The effect of steroids on macrophage activation is not confined to an effect on lysosomes, however, as evidenced by the suppression of the HMS activity which was reported in an earlier study (13). Recently, a mechanism has been postulated to explain the BCG-stimulated increase in HMS activity. Nathan et al. (16) examined the alterations of macrophage functions by mediators from lymphocytes, and reported that a fraction rich in macrophage-inhibiting factor caused a marked stimulation of HMS activity in the macrophage. Steroid suppression of the shunt stimulation by BCG may be explained by its interference with the effect of macrophage-inhibiting factor. Indeed, in their in vitro study of the mechanisms of steroid suppression of cellular immunity, Balow and Rosenthal (1) found a decrease in macrophage migration inhibition after the addition of hydrocortisone. The interference of steroid with the action of macrophage-inhibiting factor may explain the decreased number of cells found in the lungs of BCG-im-

munized animals that are also treated with steroid.

It is not known whether the steroid effects on lysosomal density, cell number, and enzyme activity are mediated by the same mechanism as the effects on HMS activity. It is possible that the steroid exerts at least two independent effects, one affecting HMS activity and one affecting lysosomal density. It is likewise possible that both effects result from a single initial event, e.g., an interference with the effect of macrophage-inhibiting factor.

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