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Participation of Lipid Peroxides in Rat Pertussis Vaccine Pleurisy. II. Leucocytes and Related Enzymes

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The peroxidation of lipids and the activities of related enzymes, such as superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), the migration of leucocytes in the pleural space, and the release of superoxide by leucocytes were studied in a rat model of *Bordetella pertussis* vaccine pleurisy.

A pleural injection of *B. pertussis* vaccine into sensitized rats was found to be associated with a transient increase of lipid peroxidation in the exudate and the serum, but the level of thiobarbituric acid-reactive substance rapidly diminished.

The diminution of lipid peroxidation was not related to the activity of GSH-Px but was related to the activity of SOD. The migration of leucocytes into the pleural cavity increased gradually and reached the maximum value 96 h after the intrapleural challenge, but the release of superoxide by leucocytes was not correlated with the number of leucocytes. The number of polymorphonuclear cells (PMN) reached the maximum value 24 h later, then gradually decreased, and mononuclear cells replaced PMN. Mononuclear cells were more numerous than PMN in the leucocyte population of the exudate in vaccine-induced pleurisy. The release of superoxide by leucocytes reached the maximum value 24 h later, then decreased gradually. Therefore, the production rate of superoxide by PMN was larger than that by mononuclear cells.

A principal component analysis of these parameters was carried out. It has been suggested that the inflammatory process of pertussis vaccine pleurisy can be divided into three phases. The first response (0—24 h) represents the acute phase response; this phase has the characteristic of a predominantly PMN population in the exudate and there is marked superoxide generation or lipid peroxidation. The second phase of the response (24—96 h) represents the delayed-type reaction; this phase has the characteristic of a predominantly mononuclear cell population in the exudate. The third phase of the response (96—144 h) represents the healing process of the inflammatory reaction.

Keywords——pertussis vaccine pleurisy; lipid peroxidation; superoxide dismutase; glutathione peroxidase; leucocyte migration; superoxide; principal component analysis

Introduction

McCord[1] reported that superoxide radicals react with hydrogen peroxide to produce hydroxyl radicals, which depolymerize purified hyaluronic acid and bovine synovial fluid. Since phagocytizing polymorphonuclear leucocytes, which are present in the synovial fluid, produce superoxide radicals (O_2^-) with attendant generation of hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH\cdot$), the reaction was suggested to reflect the *in vivo* mechanism of synovial fluid degradation in inflamed joints.[2] Halliwell and his coworkers[3] showed that activated phagocytic cells produce superoxide and hydrogen peroxide; this is important in bacterial killing by neutrophils and has been implicated in tissue damage by activated phagocytes. H_2O_2 and O_2^- are poorly reactive in aqueous solution and the damaging effects may be a consequence of the formation of more reactive species. One of the possible species is the hydroxyl radical. Hydroxyl radical is known to be an initiator of the lipid peroxidation

and a major damaging agent in the inflamed rheumatoid joints.

Fridovich[4] suggested that superoxide dismutase (SOD), when injected into an inflamed area, might minimize the damage which is caused by superoxide anion secreted by phagocytes. Huber and his coworkers[5] also suggested that exogenous CuSOD could be pharmacologically active by decreasing extracellular O_2^- levels. Tien and his coworkers suggested that O_2^- is deleterious owing to its ability to indirectly cause lipid peroxidation and proposed that this lipid peroxidation would be effectively inhibited by SOD.[6] Further, Paynter reported that decreased heart MnSOD and CuSOD activities, resulting from dietary Mn and Cu deficiencies, were both associated with increased peroxidation.[7] This concept, however, is based essentially upon *in vitro* experiments.

When unsaturated fatty acids or esters undergo oxidation, a substance is produced which develops color in a sensitive test with thiobarbituric acid (TBA). This substance is known to be malondialdehyde (MDA) or a nonvolatile precursor of MDA, and is frequently used as a measure of lipid peroxidation in both *in vitro* and *in vivo* experiments.[8]

In this study, we used the above TBA-reactive substance as an index of the lipid peroxidation. Thus, the level of TBA-reactive substance, the activities of SOD and glutathione peroxidase (GSH-Px), the number of migrated leucocytes and the leucocyte population in *Bordetella pertussis* vaccine pleurisy were determined. The production of superoxide by migrated leucocytes was also studied. The results show that the level of TBA-reactive substance and the production of superoxide by leucocytes in the exudate are related to the acute phase reaction, but are scarcely related to the retention of exudate. Diminution of the TBA reactant level is related to the increase of the SOD activity, but not to the activity of GSH-Px. Further, the increase of the TBA reactant level and the SOD activity were shown to be elicited by production of superoxide by migrated leucocytes.

Experimental

Materials——*Bordetella pertussis* vaccine was obtained from Chiba Serum Institute, Chiba. Freund's complete adjuvant was obtained from Iatron, Tokyo. Phosphotungstic acid was obtained from Merck, West Germany. Glutathione reductase and xanthine oxidase solution were obtained from Boehringer Mannheim Yamanouchi, Tokyo. Xanthine was obtained from Sigma, U.S.A. Nitroblue tetrazolium (NBT) was obtained from Tokyo Kasei, Tokyo. TBA was obtained from BDH Chemicals, Poole, England. The other chemicals were of reagent grade and were used without purification.

Induction of Pertussis Vaccine Pleurisy——Female Fischer rat were used, and pertussis vaccine pleurisy was induced as described in to the preceding paper.[9] Animals were sacrificed at 24, 48, 72, 96, 120, and 144 h thereafter by bleeding from the carotid arteries. The other procedures were the same as described in the previous report.

Determination of Lipid Peroxidation——The levels of TBA reactant in the pleural exudate supernatants and in the serum were measured according to the method of Yagi.[10] Lipids and lipids containing peroxides were precipitated by treating the exudate or serum with phosphotungstic acid, followed by the addition of TBA. The reaction product was then assayed fluorometrically (excitation at 515 nm and emission at 553 nm). The concentration of TBA-reactive substances was expressed in terms of the quantity of malondialdehyde (nmol/ml), using tetramethoxypropane as a standard.

Measurement of SOD Activity——Tfhe activities of SOD were assayed by a modification of the method of Imanari and his coworkers.[11] A reaction mixture consisted of 0.05 M carbonate buffer (pH 10.2) (2.4 ml), 3 mM xanthine (0.1 ml), 3 mM sodium ethylenediamine tetraacetic acid (EDTA-Na) (0.1 ml), 0.15% bovine serum albumin (Sigma) solution in distilled water (0.1 ml), 0.75 mM NBT solution (0.1 ml) and exudate or serum (0.1 ml). This was incubated at 25 °C for 10 min, and added to 0.1 ml of xanthine oxidase solution (0.02 U/ml). The mixture was incubated at 25 °C for 20 min. To stop the reaction, $CuCl_2$ solution was added. The reaction product was then assayed spectrophotometrically (560 nm).

Measurement of the GSH-Px Activity——The activities of GSH-Px were determined according to Lawrence and his coworkers.[12] A reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN_3, 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 E.U./ml oxidized glutathione (GSSG)-reductase, 1 mM GSH, and 0.25 mM H_2O_2 in a total volume of 1 ml. The ingredients, except the enzyme source and peroxide, were combined at the beginning of each assay. Samples (0.1 ml) were added to 0.8 ml of the above mixture and incubated for 5 min at 25 °C, before initiating the reaction by the addition of 0.1 ml of peroxide

solution. The absorbance at 340 nm was recorded for 5 min. The activity was then calculated from the slope of the lines as μmol of NADPH oxidized per minute. The blank datum (the enzyme was replaced by distilled water) was subtracted from each value.

Measurement of Protein——The amount of protein was determined by the method of Lowry and his coworkers.[13]

Measurement of the O_2^- Generation——The generation of O_2^- was measured in terms of the reduction of ferricytochrome C (horse heart type III) as described by Goldstein and his coworkers.[14]

Leucocytes (1×10^7) and 0.1 mM ferricytochrome C were incubated in the presence of opsonized zymosan for 30 min at 37 °C. The final volume of the reaction mixture was adjusted to 2.0 ml. Opsonized zymosan was prepared by incubating 50 mg of zymosan in 1 ml of freshly prepared normal rat serum for 40 min at 37 °C. After centrifugation this at $1700 \times g$ for 15 min, the opsonized particles were suspended again at a concentration of 50 mg/ml in phosphate-buffered saline. This suspension was stored at −80 °C until use.

The incubation was terminated by placing the tubes in an ice-water bath; they were then centrifuged at $700 \times g$ for 10 min at 4 °C. The absorbances of the supernatants were read at 550 nm with a spectrophotometer.

Measurement of Migrated Leucocytes——The total of number leucocytes was counted with an improved Neubauer's ruling for counting under a microscope. A differential count of the polymorphonuclear, lymphocytic and mononuclear cells was carried out by use of a May-Giemasa's stained smear preparation.

Statistical Method——The results are shown as mean values ± standard deviation (mean ± S.D.); the significance of the differences was evaluated by analysis of variance employing error variance. Further, as a method of multivaruate analysis, the principal component analysis was chosen.[15] Namely, we produced the data matrix of the observed values of all parameters. From this matrix, X, was made a correlation matrix, R. Eigen vectors, as well as eigen values, were calculated against the correlation matrix.

Results

Bordetella pertussis vaccine injected into the pleural cavity of sensitized rats was found to bring about a remarkable inflammatory reaction, which is characterized by retention of exudate and an increase of the mononuclear cell population in the pleural space. Figure 1 shows the exudate volume and the level of TBA-reactive substance in the exudate and serum. The exudate retention volume promptly increased, and became maximal from 72 to 96 h after intrapleural challenge. It then decreased gradually to 144 h. The level of TBA reactant in the exudate and the serum, on the other hand, exhibited the maximum value after 24 h, and then decreased rapidly.

Figure 2 shows the activities of SOD and the level of TBA react ant. The activity of SOD in the exudate changed in parallel with the exudate volume, and reached the maximum value 96 h after challenge, while the activity of SOD in the serum decreased temporarily at 24 h, and thereafter recovered gradually to normal levels. However, the level of TBA reactant in the exudate changed in an opposite manner to the variation of SOD activities.

Figure 3 shows the activities of GSH-Px in the exudate and the serum, and the level of TBA reactant in the exudate. The activity of GSH-Px in the exudate display peaks at 24 and

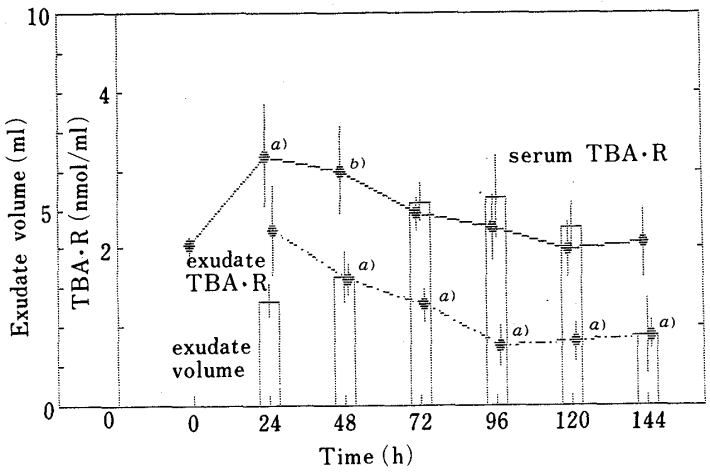

Fig. 1. Comparison between Exudate Volume and TBA Reactant Content

Time courses of exudate volume and TBA reactant contents in rat pertussis vaccine pleurisy. Correlation coefficients between exudate volume and exudate TBA reactant or serum TBA reactant are $r = -0.143$ or -0.238, respectively. Each value represents the mean ± S.D. of 5 rats. *a*) Significant difference from 0 or 24 h at $p < 0.01$. *b*) indicates $p < 0.05$.

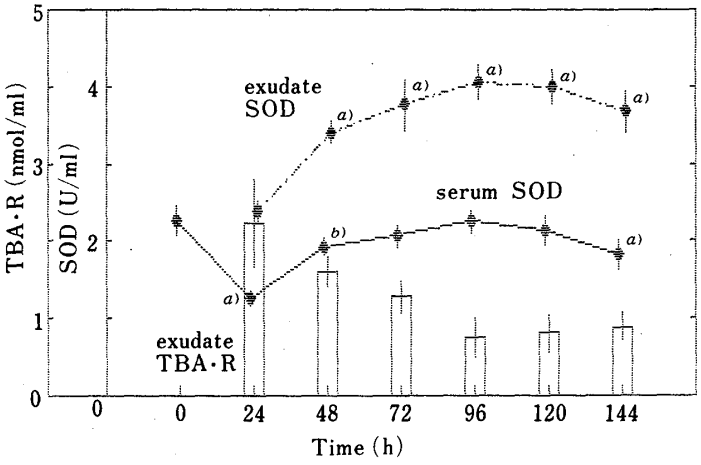

Fig. 2. Comparison between TBA Reactant Content in Exudate and SOD Activity

Time courses of SOD activity and TBA reactant content in exudate of rat pertussis vaccine pleurisy. Correlation coefficients between TBA reactant content in exudate and exudate SOD activity or serum SOD activity are −0.547 or −0.638, respectively. *a*) Significant difference from 0 or 24 h at $p<0.01$. *b*) indicates $p<0.05$.

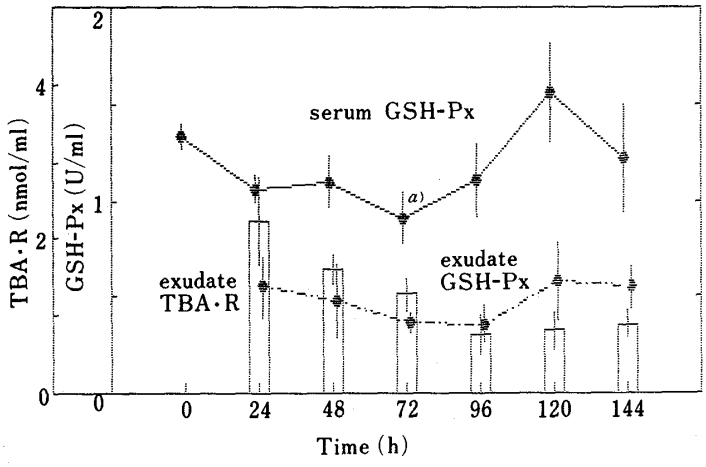

Fig. 3. Comparison between TBA Reactant Content in Exudate and GSH-Px Activity

Time courses of GSH-Px activity and TBA reactant content in exudate of rat pertussis vaccine pleurisy. Correlation coefficients between TBA reactant content in exudate and exudate GSH-Px activity or serum GSH-Px activity are each −0.119. LDH activities were shown as the inverse number of optical density. *a*) $p<0.05$.

TABLE I. Levels of TBA Reactant and Enzyme Activities in Rat Pertussis Vaccine Pleurisy

Hours	Volume (ml)	Exudate				Serum			
		TBA·R	SOD	GSH-Px	Protein	TBA·R	SOD	GSH-Px	Protein
0	—	—	—	—	—	2.04 ±0.11	2.27 ±0.18	1.33 ±0.06	56.35 ±1.26
24	2.66 ±0.41	2.23 ±0.56	2.38 ±0.14	0.55 ±0.16	45.91 ±2.52	3.19[a] ±0.64	1.26[a] ±0.10	1.06 ±0.07	63.80 ±4.26
48	3.28 ±0.63	1.60[a] ±0.20	3.41[a] ±0.14	0.48 ±0.19	44.96 ±2.38	3.00[b] ±0.54	1.93[b] ±0.10	1.10 ±0.13	56.38 ±8.83
72	5.16 ±0.47	1.28[a] ±0.21	3.76[a] ±0.34	0.36 ±0.05	40.79 ±1.72	2.44 ±0.21	2.06 ±0.15	0.91[b] ±0.13	53.12 ±2.73
96	5.31 ±1.02	0.76[a] ±0.26	4.06 ±0.22	0.36 ±0.09	37.21[b] ±7.47	2.26 ±0.42	2.25 ±0.15	1.11 ±0.19	57.03 ±3.55
120	4.55 ±0.60	0.81[a] ±0.24	3.99 ±0.23	0.58 ±0.20	45.40 ±2.12	1.99 ±0.34	2.14 ±0.20	1.56 ±0.25	73.80[b] ±7.69
144	1.78 ±0.95	0.89[a] ±0.17	3.67 ±0.27	0.55 ±0.11	44.09 ±2.98	2.07 ±0.42	1.82[a] ±0.19	1.22 ±0.28	68.76 ±4.37

Each value represents the mean±S.D. of 5 rats; TBA·R, TBA reactants are shown as nmol of MDA/ml. SOD activities are shown as U/ml. GSH-Px activities are shown as U/ml. Protein levels are shown as mg/ml. TBA-reactive substances were measured fluorometrically according to the method of Yagi. *a*) Significant difference from 0 or 24 h after challenge at $p<0.001$, *b*) indicates $p<0.05$.

TABLE II. Levels of TBA Reactant and Enzyme Activities in Exudate of Rat Pertussis Vaccine Pleurisy

Hours	Volume (ml)	Total amount in exudate				Amount of per g protein in exudate		
		TBA·R	SOD	GSH-Px	Protein	TBA·R	SOD	GSH-Px
24	2.66 ±0.41	6.06 ±2.15	6.97 ±1.13	1.47 ±0.33	126.21 ±12.57	50.7 ±14.86	53.1 ± 4.81	11.8 ± 3.00
48	3.28 ±0.63	5.31 ±1.40	11.12 ±1.77	1.56 ±0.69	148.08 ±32.64	35.8[b] ± 5.08	76.0 ± 5.49	10.6 ± 3.81
72	5.16 ±0.47	6.53 ±0.79	19.40[a] ±2.34	1.87 ±0.36	210.24[a] ±18.03	31.2[a] ± 4.31	92.6[a] ±11.35	8.9 ± 1.11
96	5.31 ±1.02	4.05[a] ±1.57	21.52[a] ±4.27	1.87 ±0.56	197.69[a] ±57.13	22.9[a] ±14.74	114.3[a] ±33.00	10.0 ± 3.52
120	4.55 ±0.60	3.73[a] ±1.45	18.25[a] ±3.16	2.57[a] ±0.64	206.51[a] ±27.55	17.7[a] ± 4.84	88.3[a] ± 8.42	12.7 ± 3.93
144	1.78 ±0.95	1.61[a] ±0.76	6.56 ±3.31	1.06 ±0.73	80.43 ±45.05	20.2[a] ± 3.39	83.4[b] ± 7.35	12.4 ± 1.87

Each value represents the mean±S.D. of 5 rats; TBA·R, TBA reactants are shown as nmol MDA or nmol/g protein. SOD activities are shown as U or U/g protein. GSH-Px activities were shown as U or U/g protein. Protein amounts are shown as mg. TBA-reactive substances were measured fluorometrically according to the method of Yagi. *a*) Significant difference from 24 h after challenge at $p<0.001$, *b*) indicates $p<0.05$.

TABLE III. Correlation Coefficients between Various Parameters in Rat Pertussis Vaccine Pleurisy

	E.V.	Exudate				Serum			
		TBA·R	SOD	GSH-Px	Protein	TBA·R	SOD	GSH-Px	Protein
Exudate volume	1								
Exudate									
TBA reactant	−0.143	1							
SOD	0.528	−0.547	1						
GSH-Px	0.221	−0.119	0.147	1					
Protein	−0.275	−0.274	0.111	0.454	1				
Serum									
TBA reactant	−0.238	0.765	−0.745	−0.200	−0.203	1			
SOD	0.418	−0.638	0.741	0.128	0.052	−0.822	1		
GSH-Px	−0.378	−0.119	−0.227	0.300	0.297	−0.003	−0.148	1	
Protein	−0.327	0.161	−0.363	0.379	0.223	0.180	−0.346	0.559	1

The alteration of the pleural exudate volume was shown to be correlated slightly with the SOD activity in the exudate. The SOD activity shows a positive correlation with the exudate volume (E.V. 0.528). The level of TBA reactant in the exudate shows a positive correlation with serum TBA·R levels (0.765), and a negative correlation with SOD activity in serum (−0.638).

120 h. On the other hand, the GSH-Px activity in the serum became maximal at 120 h. These peaks did not coincide with the peaks of the other parameters.

Tables I and II summarize the above results and Table III lists the correlation coefficients between various parameters determined in this work.

The content or amount of per g protein of TBA·R and ceruloplasmin in exudate showed similar trends to those of concentration.

The variation of the pleural exudate volume was correlated slightly to the SOD activity in the exudate (correlation coefficient $r=0.528$). The other parameters were found to be not correlated with the exudate volume. The level of TBA reactant in the exudate was shown to be correlated relatively highly to the level of TBA reactant and the activity of SOD in the serum ($r=0.765$ and -0.638, respectively). In the exudate, the activity of SOD was shown to be

correlated slightly with the level of TBA reactant ($r = -0.547$). In the serum, however, it was relatively highly correlated with the level of TBA reactant and the activity of SOD ($r = -0.745$ and 0.741 respectively). The highest correlation was found between the level of TBA reactant and the activity of SOD in the serum ($r = -0.822$).

Figure 4 shows the level of TBA reactant and the release of superoxide by leucocytes into the pleural space. The generation of superoxide became maximal at 24 h and then decreased gradually. The variation of the TBA reactant levels was parallel to the degree of superoxide generation by leucocytes.

Figure 5 shows the population of leucocytes and the number of migrated intrapleural leucocytes. The migration of leucocytes reached the maximum 96 h after challenge, and the cell population was dominated by mononuclear cells. Polymorphonuclear leucocytes predominated at 24 h, and then rapidly decreased, being replaced by mononuclear cells.

Figure 6 shows the release of superoxide by leucocytes, the number of migrated intrapleural leucocytes and the percentage of neutrophils. The alteration of superoxide generation was related to neutrophils, but not related to the number of migrated leucocytes in the exudate.

In order to see whether the levels of TBA reactant and the activities of related enzymes, such as SOD and GSH-Px, were related to the inflammatory process, we carried out a principal component analysis, (one of the methods of multivariate analysis).

The results of the principal component analysis are summarized in Figs. 7 and 8. The factor loading of each parameter for the first and second components is illustrated in Fig. 7.

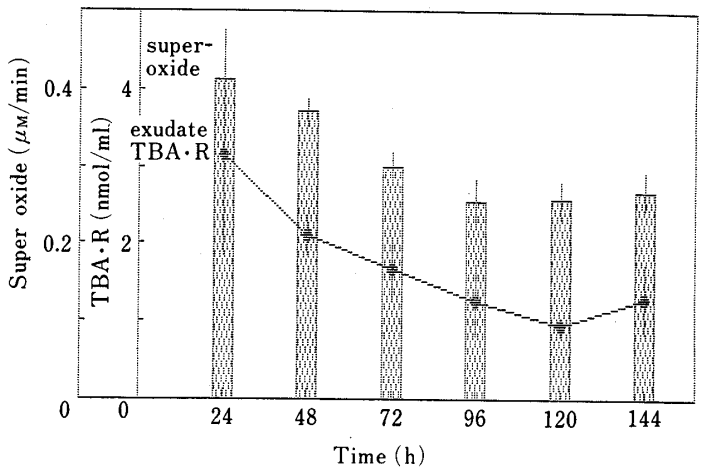

Fig. 4. Time Courses of TBA Reactant Content in Exudate and Generation of Superoxide by Migrated Leucocytes

The generation of superoxide was measured in terms of the reduction of ferricytochrome C (horse heart type III).

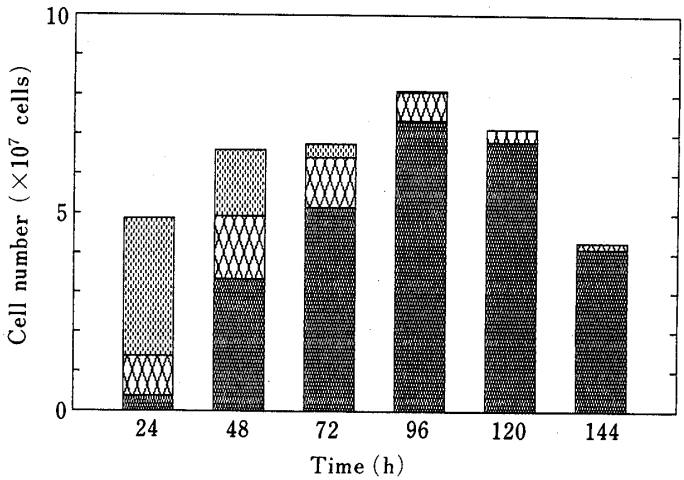

Fig. 5. Time Courses of Leucocyte Population and Leucocyte Count in the Pleural Gavity

Upper: neutrophil. Middle: lymphocyte. Lower: monocyte. The total number of leucocytes were counted with an improced Neubauer's ruling for counting under a microscope and differential counts were performed on a May-Giemsa-stained smear preparation.

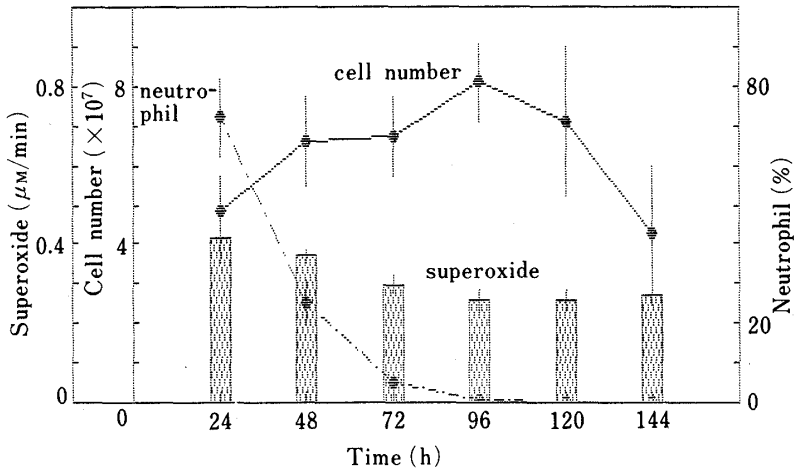

Fig. 6. Time Courses of Generation of Superoxide by Migrated Leucocytes, Leucocytes Count and the Percentage of Populated Neutrophils

The alteratio of superoxide generation was related to the predominance of neutrophils, but not to the number of migrated leucocytes in the exudate.

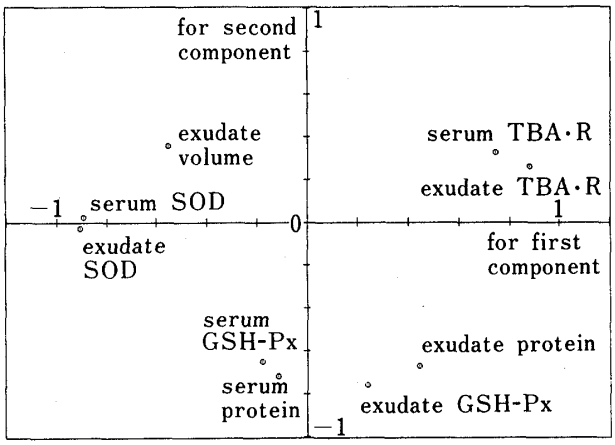

Fig. 7. Factor Loadings of Principal Component Analysis

Factor loadings are as follows: exudate SOD, −0.905, serum SOD, −0.893, exudate TBA reactant, 0.881, serum TBA reactant, 0.748 for first component; exudate protein, −0.669, serum protein, −0.719 for second component.

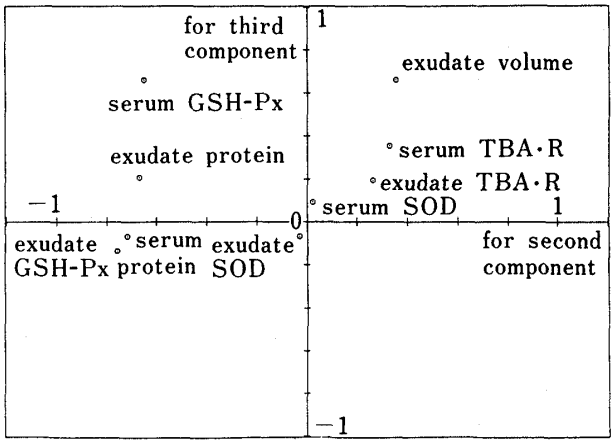

Fig. 8. Factor Loadings of Principal Component Analysis

Factor loadings are as follows: exudate volume, 0.662, serum GSH-Px, 0.659 for third component.

Figure 8 lists the factor loading of each parameter for the second and third components. The first component is the one explaining the largest portion, namely, 39.5% of the sample variance; this has high negative correlations with the activities of SOD in the exudate and the serum ($r = -0.905$ and -0.893, respectively) and a positive correlation with the level of TBA reactant in the exudate and the serum ($r = 0.881$ and 0.749, respectively). On the other hand, the volume of stored pleural exudate, which is considered to be a representative parameter in the process of inflammatory reaction, is correlated slightly with the first component. The second component, explaining 25.2% of the sample variance, has a relatively high correlation with the concentrations of protein in the exudate and the serum ($r = -0.669$ and -0.719, respectively) and the activities of GSH-Px in the exudate and the serum ($r = -0.758$ and -0.649, respectively). The third component, explaining 12.3% of the sample variance, has relatively high correlations with the volume of pleural exudate and the activity of GSH-Px in

the serum ($r=0.662$ and 0.659, respectively).

Discussion

Evidence has been accumulated in recent years that the physiological function of SOD is the protection of tissues from free radicals. Thus Michelson and his coworkers examined the anti-inflammatory activities of eighteen SOD's from different sources, by use of the carrageenin-induced rat paw edema, the adriamycin-induced edema and adjuvant-induced arthritis. It has been found that SOD's from different sources show large differences in biological activity in all of the above models.[16] Yoshikawa *et al.* used the TBA reactant to examine the damage induced by lipid peroxidation and the protection by SOD (from bovine liver: Orgotein) in rat adjuvant arthritis. It was reported that TBA reactant was reduced significantly by the injection of SOD.[17] GSH-Px is also known to be effective in the protection of animal cells against peroxidative damage.[18] There are few papers, however, which describe the variation in the activities of these enzymes at inflammatory sites and in the serum of inflamed animals. The superoxide-generating capacity of activated phagocytic cells at inflammatory sites has rarely been documented.

In this study, we examined the level of TBA reactant, and the activities of SOD and GSH-Px, and determined the number of migrated leucocytes and the leucocyte population. The production of superoxide by migrated leucocytes was also studied. The levels of TBA reactant in the exudate and serum showed peak value at 24 h, and rapidly decreased thereafter. A similar trend was observed in total amount or amount per g protein of TBA reactant levels. The generation of superoxide by the migrated leucocytes was parallel to the level of TBA reactants, and it was shown to increase rapidly and then decrease slowly. On the other hand, the activity of SOD in the serum increased slowly and recovered to normal levels. The activity of GSH-Px in the exudate had two peaks; these peaks did not coincide with those of any other parameters. Further, the activity of GSH-Px was not related to the level of TBA reactants.

From the above facts we conclude that the lipid peroxidation is elicited indirectly through the generation of superoxide by leucocytes that migrated into the pleural space, and the diminution of lipid peroxidation may be caused by the increase of SOD activity or depression of the ability to generate superoxide of the migrated leucocytes. GSH-Px has been suggested to be unimportant in the diminution process of lipid peroxidation. The above conclusion is consistent with those reported by Paynter[7] and Kellog and Fridovich.[19]

The migration of leucocytes into the pleural cavity increased gradually and reached the maximum value at 96 h after challenge, but the release of superoxide by leucocytes was not correlated with the number of leucocytes. Therefore, the generation of superoxides by leucocyte was considered to be not related directly to the cell population in the exudate. The degree of superoxide generation has in fact been found to be dependent on the maturation rate of the migrated leucocytes, while the rate of superoxide generation by polymorphonuclear cell (PMN) has been found to be higher than that by mononuclear cells. It therefore seems that the migrated leucocytes show a distinct differences in the generation of superoxide.

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References

1) J. M. McCord, *Science.*, **185**, 529 (1974).
2) T. Richardson, *J Pharm. Pharmacol.*, **28**, 666 (1976).

3) B. Halliwell, J. M. C. Gutteridge, and D. Blake, *Phil, Trans. R. Soc. Lond.*, **311**, 659 (1985).
4) I. Fridovich, *Annu. Rep. Med. Chem.*, **10**, 257 (1975).
5) S. Carson, E. E. Vogin, W. Huber, and T. L. Schulte, *Toxicol. Appl. Pharmacol.*, **26**, 184 (1973).
6) M. Tien, B. A. Svingen, and S. D. Aust, *Fed. Proc.*, **40**, 179 (1981).
7) D. I. Paynter, *Biol. Trace Element Res.*, **2**, 121 (1980).
8) W. A. Pryor and J. P. Stanley, *J. Org. Chem.*, **40**, 3615 (1975).
9) Y. Yuda, J. Tanaka, F. Hirano, and H. Kitagawa, *Chem. Pharm. Bull.*, **36**, 2691 (1988).
10) K. Yagi, *Biochem. Med.*, **15**, 212 (1976).
11) T. Imanari, M. Hirota, and M. Miyazaki, *Igaku No Ayumi.*, **101**, 496 (1977).
12) R. A. Lawrence and R. F. Burk, *Biochem. Biophys. Res. Commun.*, **71**, 952 (1976).
13) O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) G. Witz, N. J. Lawrie, M. A. Amoruso, and B. D. Goldstein, *Chem. Biol. Interact.*, **53**, 13 (1985).
15) M. Kendall, "Multivariate Analysis," Charles Griffin and Co., London, 1975.
16) A. M. Michelson, G. Jadot, and K. Puget, "The Biological Role of Reactive Oxygen Species in Skin," ed. by O. Hayaishi, S. Imamura, and Y. Miyachi, University of Tokyo Press, Tokyo, 1987, pp. 199—210.
17) T. Yoshikawa, H. Tanaka, and M. Kondo, *Biochem. Med.*, **33**, 320 (1985).
18) H. Sies (ed.), "Oxidative Stress," Academic Press, Inc., London, 1985.
19) E. W. Kellog III. and I. Fridovich, *J. Biol. Chem.*, **250**, 8812 (1975).