

Effect of Azelastine Hydrochloride on Macrophage Chemotaxis and Phagocytosis *in Vitro*

MITSUO HONDA, KAZUNORI MIURA and TOMIO TANIGAWA

Dept. of Pathology, Kumamoto University Medical School, Kumamoto, Japan

Azelastine, a newly synthesized anti-allergic agent, was tested for its effects on guinea pig macrophage chemotaxis and phagocytosis. As specific macrophage chemo-attractants, we used macrophage chemotactic factors *a* and *c*; separated and highly purified from inflamed skin sites.

Macrophage chemotaxis induced by skin extract or chemotactic factors was significantly suppressed by a low concentration of the agent (1 µg/ml); the effect was dose-dependent. The inhibition of chemotaxis was reversible, because chemotactic activity was restored when the agents was removed by washing cells before chemotactic assay. Inactivation of chemotactic factors was not detected by mixing azelastine and factors *a* and *c*. Azelastine may directly interact with macrophages to decrease their chemotactic responsiveness.

β-Glucuronidase activity in the medium and macrophages after phagocytosis of polystyrene latex particles was not affected by this agent at concentrations ranging from 1 to 10 µg/ml. The phagocytosis of latex particles or sheep red blood cells opsonized with IgG antibodies (EA) and anchoring of macrophages to substrate were not inhibited and azelastine did not damage the macrophages as determined by lactate dehydrogenase (LDH) release assay.

Key words: anti-allergic drug; macrophage chemotaxis; macrophage chemotactic factor; macrophage phagocytosis.

Accepted for publication 23 August 1981

4-(*p*-chlorobenzyl)-2[*N*-methylperhydroazepinyl-(4)]-1-(2)-phthalazinone hydrochloride (azelastine), one of several phthalazinone derivatives, reportedly (31) has an anti-allergic effect; there are several reports on its inhibition of bronchial asthma and immediate or delayed allergic reactions (13). Recently, we documented in guinea pigs that increased vascular permeability in the reversed passive Arthus reaction (RPA) was significantly suppressed by this agent (27).

There is increased interest in the role of macrophage chemotaxis and phagocytosis in normal host defense and in the aberrations of this function in disease states or secondary to pharmacologic agents. Despite the significance of macrophages in inflammation, factors in-

ducing their selective migration to inflammatory sites have not yet been completely elucidated. To clarify the mediation of macrophage chemotaxis *in vivo*, we previously induced hypersensitivity reactions in guinea pigs by injecting dinitrophenyl-ascaris extract or purified protein derivative (PPD); three types of macrophage chemotactic factors were separated from the skin site (7, 8, 9, 13, 14, 15).

We now demonstrate the *in vitro* effect of azelastine on macrophage chemotaxis, using macrophage chemotactic factors and phagocytosis, which are the important functions of macrophages in inflammation, host resistance and the immune response.

MATERIAL AND METHODS

Preparation of inflamed skin extract

Delayed type hypersensitivity reaction was induced as follows (24): male Hartley strain guinea pigs, weighing 300–350 g, were immunized by intradermally (i.d.) injecting 1.6 mg of BCG (Japan BCG Corporation, Tokyo, Japan). Six weeks later, they were i.d. injected with 10 µg (500 IU) of PPD (Ministry of Agriculture, Veterinary Laboratory, Weybridge, England) per site at 10 sites on the shaved back; after 24 h, the inflamed skin sites showing maximal macrophage reaction were excised, frozen at -80°C , cut into approximately 50 µm thick slices, and powdered (10). Extraction was with 0.067 M phosphate buffer (pH 7.2).

Separation and purification of macrophage chemotactic factors a and c

From the supernatant fluid, factor *a* or *c* was separated by Sephadex G-100 gel filtration and then purified by column chromatography with DEAE-Sephadex A-50 and CM-Sephadex C-50. This was followed by immunoabsorbent chromatography using anti-guinea pig IgG antibody (13, 14, factor *a*) or isotachopheresis (factor *c*). As previously described factor *a* was generated from IgG, its molecular weight (m.w.) was about 150,000 (7, 14, 15). Factor *c* existed in the form of complexes comprised of macrophage chemotactic lymphokines (1, 26) and exuded serum protein, the m.w. of the complex was about 110,000 (7, 14).

Test drug

Azelastine hydrochloride was obtained through the courtesy of Eisai Co., Ltd., Tokyo and Asta-Werke AG, W. Germany. Experiments were performed at concentrations of 0, 0.1, 1, 10, and 20 µg/ml in 0.15 M phosphate buffered saline (PBS, pH 7.2).

Chemotactic assays

Chemotactic activity for macrophages was determined by a modification of Boyden's method, using a Nuclepore filter (pore size 5.0 µm, thickness 15 µm, General Electric Company, New York, U.S.A.) (13). The chambers were incubated at 37°C for 90 min in a 5% CO_2 atmosphere. The cells, which had migrated through the filter to its lower surface, were Giemsa-stained and counted on five randomly selected high-power fields (10×40).

Guinea pig macrophages were collected from the peritoneal cavity 4 days after instillation of mineral oil and 5×10^5 cells/ml were suspended in RPMI 1640 medium (pH 7.2, GIBCO, Grand Island, N.Y., U.S.A.), containing 10% heat-inactivated fetal calf serum (Microbiological Associates Inc., Bethesda, Md., U.S.A.) and 5 U heparin (Sigma, St. Louis, Mo., U.S.A.).

Latex phagocytosis experiments

These were essentially by the method of Kvarstein (19). Equal volumes (0.5 ml) of PBS containing various concentrations of azelastine and autologous plasma were admixed with 1 ml of macrophage suspension (1×10^7 cells/ml in PBS containing 25% plasma).

After 15 min, 37°C pre-incubation of tubes containing macrophages in the absence or presence of azelastine, 0.1 ml of 1% polystyrene latex particles (Dow Chemical Company, Midland, Mich., U.S.A.) in PBS, prepared by the method of Kvarstein (18), was added. The tubes were then incubated at 37°C for 60 min in a constantly revolving rotor. Superficially attached particles were removed by trypsin-DNase treatment of macrophages after incubation with particles by the method of Ultrich & Zilbersmit (28). The effect of azelastine on the uptake of latex particles was determined on smears stained by the method of Kvarstein (19).

Enzyme assays

The cells were then spun for 10 min at 450 g and the supernatants were removed and used

No cells remained in the supernatant after spinning. The cells at the bottom were re-suspended in a mixture of 2.0 ml of 0.34 M sucrose and 0.1 ml of 2% triton and incubated at 37°C for 10 min. After 20 min centrifugation at 15,000 *g* in the cold, the supernates were used for enzyme assay.

β -Glucuronidase was the lysosomal enzyme marker (12), the activity was measured by the method of Kato et al. (17). Supernatant fluid (0.1 ml) was added to a tube containing 0.1 ml of substrate (20 mM *p*-nitrophenol glucuronide in 0.2 M acetate buffer, pH 4.5) and 2.8 ml of 0.1 M acetate buffer (pH 4.5). After 2 h incubation at 37°C, the reaction was stopped by adding 1.0 ml of 0.1 N NaOH. Hydrolyzed *p*-nitrophenol was measured by spectrophotometry at 400 nm.

Lactate dehydrogenase (LDH) activity, as a marker of cytoplasmic enzyme, was determined by the method of Wroblewski & La Due (30). To a tube containing 0.5 ml of substrate solution and pyruvic acid and NADH, previously incubated at 37°C for 3 min, was added 0.05 ml of supernatant fluid. After 10 min incubation at 37°C, the reaction was stopped by adding 5.0 ml of 0.1 N HCl; the activity was read at 560 nm. The percentage of total LDH activity in the medium was obtained by dividing the LDH activity in the medium by the total activity of the cells.

Immune phagocytosis assays

After pre-incubation of the drug and the macrophages as described above without plasma, immune phagocytosis was evaluated by incubating cells with Fc rosetts (2, 22) for 60 min at 37°C by the method of Lotem & Sachs (21). Cells interiorizing one or more sheep red blood cells opsonized with IgG antibodies (EA) were scored as positive.

Macrophage anchoring assays

The attachment of macrophages to the surface of a petri dish was quantified from counts of the numbers of cells per unit area of its surface, after pre-incubation of the drug and the cells as

described above. The morphology of the attached macrophages on the petri dish surface was also evaluated by phase contrast microscopy and the proportion of spreading macrophages was estimated for the attached cells (5, 20).

The results were analyzed by the Student's *t*-test and by regression analysis; *P* values of 0.05 or less were considered to be significant.

RESULTS

Effect of azelastine on in vitro macrophage chemotaxis

The effect of macrophage exposure to various concentrations of azelastine on their chemotactic responsiveness is shown in Table 1. Inflamed skin extract, at absorbance of 1.0 at 280 nm, was placed in the lower compartment of the chamber since such concentration of the extract exhibited a distinct macrophage chemotactic activity. Macrophages were pre-incubated at 37°C for 60 min in the presence of the indicated azelastine concentrations before placing them in the upper compartment of the chamber. While no significant effect was seen at 0.1 μ g/ml azelastine, an increase in the drug concentration brought about a significant,

Table 1

Effect of azelastine on macrophage chemotactic activity to inflamed skin extract*

Concentration of azelastine (μ g/ml)	No. migrated macrophages (means $\pm$ SD)**	<i>P</i>
None	105 $\pm$ 4.4	N.S.
0.1	106 $\pm$ 10.2	
1.0	44 $\pm$ 5.9	0.005
10.0	30 $\pm$ 3.7	
20.0	15 $\pm$ 4.4	0.025

* Extracted from 24-h skin lesions from BCG-sensitized, PPD-challenged guinea pigs.

** From 5 experiments.

Chemotactic assay was performed at the same concentration (absorbancy 1.0 at 280 nm) of extract.

Table 2

Effect of azelastine on macrophage chemotactic factors *a* and *c*

Lower chamber	Number of migrated macrophages (means $\pm$ SD), upper chamber	
	Cells	Cells + azelastine*
Factor <i>a</i> **	224 $\pm$ 25	91 $\pm$ 13
Factor <i>a</i> ** (with azelastine)*	205 $\pm$ 18	—
Factor <i>c</i> **	285 $\pm$ 39	132 $\pm$ 15
Factor <i>c</i> ** (with azelastine)*	237 $\pm$ 23	—

* Preincubated with 1 μ g/ml azelastine for 60 min at 37°C.

** Absorbancy 0.045 at 280 nm.

Values are the mean $\pm$ SD of 5 experiments.

Table 3

Effect of azelastine on macrophage phagocytosis

Concentration of azelastine (μ g/ml)*	No. of latex particles ingested**			No. of EA ingested**		
	0-4	5-20	>20	0	1-5	>5
	%			%		
0	21	41	38	17	28	56
1	19	40	41	13	32	55
10	17	36	46	19	26	54

* During 15 min, 37°C pre-incubation.

** Latex particles or EA were present during 60 min incubation at 37°C.

Values are the mean of 4 experiments.

Table 4

Effect of azelastine on β -glucuronidase activity of phagocytosing macrophages

Concentration of azelastine (μ g/ml)	Enzyme activity	
	in medium (μ M/h/mg protein)	in the cells (μ M/h/mg protein)
0	9.49 $\pm$ 0.34	533.8 $\pm$ 19.12
1	8.87 $\pm$ 0.46	474.0 $\pm$ 13.42
10	9.10 $\pm$ 0.52	482.73 $\pm$ 6.84

Values are the mean $\pm$ SD of 4 experiments.

dose-related decrease in mean chemotactic activity. A clear negative correlation ($r = 0.774$) was found when chemotactic activity, indicated by the number of migrated cells, was plotted against the drug concentration.

On the other hand, when the cells were incubated for 60 min at 37°C in the presence of 1 μ g/ml azelastine and then washed twice with 0.15 M PBS, the mean chemotactic activity was 97, close to the control value of 105. This suggests that the observed agent-induced inhibition of macrophage chemotaxis may be reversible (data not shown).

When purified macrophage chemotactic factors *a* and *c* were tested after 60 min, 37°C incubation with 1 μ g/ml azelastine, the macrophage chemotactic activity decreased slightly (Table 2). Macrophages incubated in the presence of azelastine clearly manifested decreased chemotaxis, suggesting that azelastine at the concentration used, may not affect the activity of chemotactic factors *a* and *c*, but may rather exert a direct inhibitory effect on the macrophages.

Effect of azelastine on macrophage phagocytosis

After 60 min incubation in the presence of latex particles or EA, there was no marked difference in phagocytosis, irrespective of the azelastine concentrations used (Table 3).

As shown in Table 4, the mean β -glucuronidase activity of latex-phagocytosing macrophages was not affected by azelastine at the concentrations tested. This suggests that the drug does not influence the lysosomal enzyme activity in phagocytosing macrophages.

To determine whether azelastine is toxic to the cells, the LDH activity was studied in the incubation medium and the cells. The drug had no effect on the release of LDH from phagocytosing macrophages, nor did it influence the cytoplasmic enzyme activity at the tested concentrations (Table 5).

Effect of azelastine on macrophage anchoring

Similar numbers of control macrophages and macrophages with various concentrations of

Table 5
Effect of azelastine on lactate dehydrogenase activity of phagocytosing macrophages

Concentration of azelastine ($\mu\text{g/ml}$)	Enzyme activity		Percent of total enzyme activity in medium
	in medium (U*)	in cells (U*)	
0	497 $\pm$ 22.5	2090.5 $\pm$ 35	19.2
1	507.5 $\pm$ 13.5	2114.5 $\pm$ 49.3	19.4
10	525.8 $\pm$ 13.4	2068.8 $\pm$ 61.6	20.2

* Estimated by the method of Wroblewski & La Due (30) after measuring the absorbancy of test samples and control serum at 560 nm.

Values are the mean $\pm$ SD of 4 experiments.

azelastine became attached to the dishes, and the azelastine-treated macrophages spread in the normal manner. The anchoring of the azelastine-treated macrophages to the surface of the plastic petri dish appeared to be grossly normal.

DISCUSSION

The present findings demonstrate that, compared to other drugs (4, 6, 25), a small amount of azelastine (1 $\mu\text{g/ml}$) inhibits macrophage chemotaxis induced by skin extract from BCG-sensitized, PPD-challenged hypersensitivity skin reaction site or by purified macrophage chemotactic factors *a* or *c*, separated from the extract (7, 8, 9, 13, 14, 15). Furthermore, inhibition was found to be dose-dependent (Table 1). The *in vitro* suppressive effect disappeared when the drug was washed from the cells prior to testing, suggesting that the inhibition may be reversible. The inhibition of chemotaxis by azelastine was not due to changes in the stickiness or spreading of the macrophages. Azelastine exhibited no inactivating effect on chemotactic factors *a* and *c*, while a marked decrease in chemotaxis was detected when the macrophages were preincubated in the presence of azelastine before assay (Table 2). This suggests that the drug may act directly on the macrophages to render them incapable of responding appropriately to the applied chemotactic factors, e.g. the agent may bring about some cell surface alterations or change intracellular chemotactic response.

Many pharmacologic agents with anti-inflammatory effect are capable of inhibiting neutrophil chemotaxis (3, 23, 29). However, only few pharmacologic agents that suppress macrophage chemotactic activity have been described (4, 6, 23). Snyderman & Stahl (25) reported that heparin at more than 10 units/ml markedly suppressed macrophage chemotaxis. Borel & Feurer (4) showed that phenylbutazone and sodium salicylate suppressed rabbit macrophage chemotaxis while they had little effect on neutrophil chemotaxis. Fisher et al. (6) noted the inhibition of human macrophage chemotaxis by aspirin, whereas neutrophil chemotaxis was unaffected. The mechanism of such chemotaxis inhibition by these agents remains to be ascertained.

In studies of phagocytosis it is often difficult to distinguish between an intracellular particle and one which is adherent to the cell surface but not ingested. To detect the intracellular particles, adherent polystyrene latex particle was removed by trypsin-DNase treatment of the cells (28) and adherent EA was lysed without damage to the macrophages (21). From the present experiments, it is clear that azelastine did not affect the phagocytosis of latex particles or EA by macrophages and that this agent did not affect lysosomal or cytoplasmic enzyme activity in the phagocytosing macrophages or the release of enzymes into the medium. Our results were similar to those of Hawkins (11), who reported the lysosomal enzyme release from neutrophils to be relatively resistant to drug

inhibition by salicylates, cromoglycates and diethylcarbamazine.

We found inhibitory effect on RPA and the increased vascular permeability in RPA was significantly suppressed when azelastine was given to guinea pigs orally in a comparatively large dose (27). Katayama et al. (16) also found that passive cutaneous anaphylaxis in guinea pigs was inhibited by azelastine.

The effect of azelastine on delayed allergic reactions, on the other hand, seems to be controversial. One has been shown to be inhibitory (unpublished results), whereas others seem to have no effect (16). Major reasons which account for the difference between those two studies may result from that of methodology of production of allergic reactions.

The effects of azelastine on macrophage function may indicate that the beneficial action of azelastine in treatment of allergic reaction is due to decreased chemotaxis of macrophages in the lesion, while the absence of any effect on phagocytosis and lysosomal enzymes shows that the cells still have the ability to ingest and destroy the bacteria and other antigenic substance. Further studies are needed to investigate the effects of azelastine on other leukocytes such as neutrophils, eosinophils and lymphocytes.

ACKNOWLEDGEMENTS

We are grateful to Prof. H. Hayashi for his valuable suggestions and critical reading of the manuscript. We also thank Assist. Prof. A. Iwamasa for performing enzyme assays and Eisai Co., Ltd., Tokyo (Japan) and Asta-Werke AG, Bielefeld (W. Germany) for providing the azelastine.

REFERENCES

- Altman, L. C., Snyderman, R., Oppenheim, J. J. & Mergenhagen, S. E.: A human mononuclear leukocyte chemotactic factor: Characterization, specificity and kinetics of production by homologous leukocytes. *J. Immunol.* **110**, 801–810, 1973.
- Bianco, C., Patrich, R. & Nussenzweig, V.: A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. I. Separation and characterization. *J. Exp. Med.* **132**, 702–720, 1970.
- Björkstén, B. & Quie, P. G.: Inhibition of human neutrophil chemotaxis in vitro by phenothiazines and related compounds. *Infect. Immun.* **14**, 315–317, 1976.
- Borel, J. F. & Feurer, C.: Chemotaxis of rabbit macrophage in vitro: inhibition by drugs. *Experientia* **31**, 1437–1441, 1975.
- Crowley, C.A., Curnutte, J. T., Rosin, R. E., Andre-Schwartz, J., Gallin, J. I., Klempner, M., Snyderman, R., Southwick, F. S., Stossel, T. P. & Babior, B. M.: An inherited abnormality of neutrophil adhesion. Its genetic transmission and its association with a missing protein. *N. Engl. J. Med.* **302**, 1163–1168, 1980.
- Fisher, T. J., Gard, S. E., Rachelefsky, G. S., Klein, R. B., Borut, T. C. & Steihm, E. R.: Monocyte chemotaxis under agarose: Defects in atopic disease, aspirin therapy and mucocutaneous candidiasis. *Pediatr. Res.* **14**, 242–246, 1980.
- Hayashi, H. & Ishimaru, Y.: Morphological and biochemical aspects of adhesiveness and dissociation of cancer cells. *Int. Rev. Cytol.* **70**, 201–277, 1981.
- Hayashi, H., Hirashima, M., Honda, M. & Ishida, M.: The mediation of macrophage reaction in inflammation, with special reference to IgG-derived chemotactic factor. *Acta Pathol. Jpn.* **28**, 677–688, 1978.
- Hayashi, H., Honda, M., Ishida, M. & Hirashima, M.: The IgG-derived macrophage chemotactic factor in hypersensitivity reaction. In Kokubu, Y. & Kobayashi, N. (eds.): *Phagocytosis*, pp. 53–68. University of Tokyo Press, Tokyo, 1978.
- Hayashi, H., Miyoshi, H., Nitta, R. & Udaka, K.: Proteolytic mechanism in recurrence of Arthus-type inflammation by thiol compounds. *Br. J. Exp. Pathol.* **43**, 564–573, 1962.
- Hawkins, D.: Neutrophilic leukocytes in immunologic reactions in vitro. III. Pharmacologic modulation of lysosomal constituent release. *Clin. Immunol. Immunopathol.* **2**, 141–152, 1974.
- Hawkins, D. & Peeters, S.: The response of polymorphonuclear leukocytes to immune complexes in vitro. *Lab. Invest.* **24**, 483–491, 1971.
- Honda, M., Hirashima, M., Nishiura, M. & Hayashi, H.: A macrophage chemotactic factor sharing common antigenicity with immunoglobulin G from DNP-ascaris extract-induced skin lesion in guinea pig. *Virchows Arch. B. Cell Pathol.* **27**, 317–333, 1978.
- Honda, M., Kuratsu, J. & Miura, K.: In vivo macrophage chemotactic factors, with special reference to chemotactic lymphokine. *Igaku no Ayumi* **109**, 12–19, 1979 (in Japanese).
- Ishida, M., Honda, M. & Hayashi, H.: In vitro macrophage chemotactic generation from serum immunoglobulin G by neutrophil neutral seryl protease. *Immunology* **35**, 167–176, 1978.
- Katayama, S., Akimoto, N., Shionoya, H., Morimoto, T., Katoh, Y. & Suzuki, N.: Anti-allergic effect of azelastine hydrochloride on immediate type hypersensitivities in vivo and in vitro. *Arzneim.-Forsch./Drug Res.* (In press).
- Kato, K., Yoshida, K., Tsukamoto, H. & Nobunaga, M.: Synthesis of p-nitrophenyl β -D-glucopyranosiduronic acid

- and its utilization as a substrate for the assay of β -glucuronidase activity. *Chem. Pharm. Bull. (Tokyo)* **8**, 239–244, 1960.
18. Kvarstein, B.: A methodological study of human leukocyte adhesiveness to glass beads. *Scand. J. Clin. Lab. Invest.* **23**, 259–270, 1969.
19. Kvarstein, B.: The effect of temperature, metabolic inhibitors, and EDTA on phagocytosis of polystyrene latex particles by human leukocytes. *Scand. J. Clin. Lab. Invest.* **24**, 271–277, 1969.
20. Leonard, E. J. & Skeel, A.: A serum protein that stimulates macrophage movement, chemotaxis and spreading. *Exp. Cell. Res.* **102**, 434–438, 1976.
21. Lotem, J. & Sachs, L.: Genetic dissection of the control of normal differentiation in myeloid leukemic cells. *Proc. Natl. Acad. Sci. USA* **74**, 5554–5558, 1977.
22. Miyama, M., Kuribayashi, K., Yodoi, J., Takabayashi, A. & Masuda, T.: Immunological properties of Fc receptor on lymphocytes. I. Functional differences between Fc receptor-positive and negative lymphocytes in humoral immune responses. *Cell Immunol.* **35**, 253–265, 1978.
23. Rivkin, I., Foschi, G. V. & Rosen, C. H.: Inhibition of in vitro neutrophil chemotaxis and spontaneous motility by anti-inflammatory agents. *Proc. Soc. Exp. Biol. Med.* **153**, 236–240, 1976.
24. Shimokawa, Y.: A lymphocyte chemotactic peptide from the skin reaction site with delayed hypersensitivity induced by purified protein derivative in guinea pigs. *Kumamoto Med. J.* **32**, 67–72, 1979.
25. Snyderman, R. & Stahl, C. E.: Chemotaxis of mononuclear leukocytes. In Sorkin, E. (ed.): *Chemotaxis, its biology and biochemistry*, pp. 284–295. Karger, Basel, 1974.
26. Snyderman, R., Altman, L. C., Hausman, M. S. & Mergenhagen, S. E.: Human mononuclear leukocyte chemotaxis: A quantitative assay for humoral and cellular chemotactic factors. *J. Immunol.* **108**, 857–860, 1972.
27. Tanigawa, T., Honda, M. & Miura, K.: Effect of azelastine hydrochloride on vascular permeability in hypersensitivity reaction skin site in guinea pigs. *Arzneim.-Forsch./Drug Res.* (Accepted for publication).
28. Ultrich, F. & Zilbersmit, D. B.: Release from alveolar macrophages of an inhibitor of phagocytosis. *Am. J. Physiol.* **218**, 1118–1127, 1970.
29. Ward, P. A.: The chemosuppression of chemotaxis. *J. Exp. Med.* **124**, 209–225, 1966.
30. Wroblewski, F. & La Due, J. S.: Lactic dehydrogenase activity in blood. *Proc. Soc. Exp. Biol. Med.* **90**, 210–213, 1955.
31. Zechel, H. J., Brock, N., Leuke, D. & Achterrath-Tuckermann, U.: Pharmaceutical and toxicological properties of azelastine, a novel antiallergic agent. *Arzneim. Forsch./Drug Res.* **31**, 1184–1193, 1981.

Address:

M. Honda
Dept. of Pathology
Kumamoto University Medical School
Kumamoto
Japan