Antigenicity of Nitrogen Mustard-treated Guinea Pig Serum to Guinea Pigs

by

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Received for publication Jan. 13, 1964

One of the characteristic properties of the radiomimetic alkylating agents is that their cytotoxic action is much more pronounced when the compounds carry more than one alkylating group per molecule[2)8)26)]. This property led GOLDACRE, LOVELESS & ROSS (1949)[10)] to advance a hypothesis that the compounds join in vivo chromosome threads together, resulting in chromosome breaks in subsequent mitosis. ALEXANDER, SWARCBORT & STACEY (1959)[2)] performed an experimental study to evaluate on this hypothesis and showed that when nucleoprotamine from herring sperm was brought into reaction with the alkylating agents, intermolecular reaction occurred, in which two groups of different DNA molecules were linked[1)]. These characteristics of the alkylating agents seem to suggest that the compounds might change immunological properties of protein or nucleoprotein.

This investigation was undertaken to examine whether the alkylating agents would play a role of hapten and effect some immunologically detectable changes in protein. Guinea pig serum was in vitro brought into reaction with Nitrogen Mustard (HN2) under a condition of physiological pH and temperature. With the complex of HN2 and guinea pig serum, guinea pigs were sensitized and their immunological responses were analysed with skin test, systemic anaphylaxis and passive cutaneous anaphylaxis.

EXPERIMENTAL

MATERIALS AND METHODS

I) Materials

1) $NaHCO_3$-H_2CO_3 buffer : 0.75M-$NaHCO_3$ solution saturated with CO_2 to a pH of 7.5 and stored in dark and cold.

2) Nitrogen Mustard (HN2), 2, 2'-dichloro-N-methylamine hydrochloride : Kindly supplied by Dr. Y. SAKURAI, Cancer Chemotherapy Information Center, Tokyo Sasaki Institute and stored in a desiccator.

3) Crystalline Bovine Serum Albumin (BSA) 2X : Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

4) Crystalline Egg White Albumin (Ea) 5X : Obtained from SIGMA Chemical Company, St. Louis, Missouri.

II) Preparation of Antigens

1) HN2-Guinea Pig Serum Complex (HN2-GPS Compl.)

Three guinea pigs each weighing about 700 gm were bled from jugular artery. The blood samples were allowed to clot at 5°C overnight. The sera from the three guinea pigs were pooled together and used as "guinea pig serum" throughout the experiment. A part of the "guinea pig serum" was diluted with physiological salt solution to adjust its total protein concentration to 3%, Hitachi refractometer being used to measure protein concentration throughout this study. On the other hand, nitrogen mustard hydrochloride 115.5 mg was dissolved in 3 ml of distilled water and one equivalent of NaOH was added to liberate the base. This solution of a pH of 7.0 was added slowly to 20 ml of the diluted guinea pig serum and mixed by gentle shaking. Total volume of this mixture was made 30ml with physiological salt solution. Final total protein concentration and HN2 concentration of the mixture were 2% and 2×10^{-2}M respectively (Fig. 1). After adjusting to pH 7.5 with bicarbonate buffer, the mixture was left in an incubator at 37°C for 24 hours. During the incubation the pH of this mixture was often tested with Phenol Red and Brom Thymol Blue pH test papers to keep the same level of pH by adding bicarbonate buffer. After 24 hours of incubation the complex of HN2 and guinea pig serum (HN2-GPS compl.) was stored frozen at −20°C. All these procedures were done as aseptically as possible.

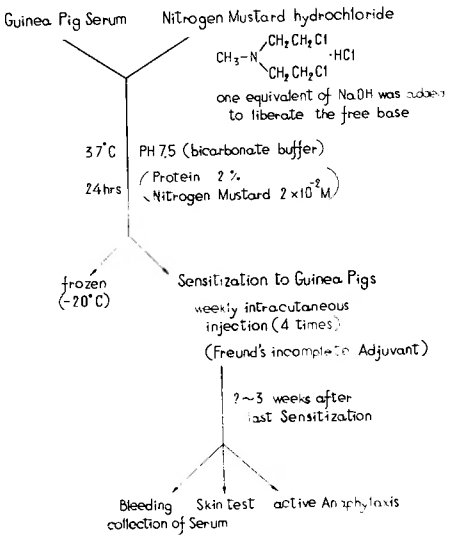


Fig. 1 Preparation and sensitization of HN2-GPS compl.

2) Untreated Guinea Pig Serum (Untreated GPS)

The rest of the "guinea pig serum" was diluted with physiological salt solution to adjust its total protein concentration to 2%. The diluted guinea pig serum was adjusted to a pH of 7.5 with bicarbonate buffer and left in an incubator at 37°C for 24 hours and stored frozen at −20°C.

3) HN2-Rabbit Serum Complex (HN2-RS Compl.), HN2-Bovine Serum Albumin Complex (HN2-BSA Compl.) and HN2-Egg Albumin Complex (HN2-Ea Compl.)

Each of crystalline bovine serum albumin 2X, and crystalline egg white albumin 5X was dissolved in physiological salt solution to give a solution a total protein concentration of 3%. Rabbit serum obtained from a normal rabbit weighing about 3 kg, the bovine serum albumin and the egg albumin were treated with HN2 in the same way as HN2-GPS compl., to make HN2-RS compl., HN2-BSA compl. and HN2-Ea compl. respectively.

4) Untreated Rabbit Serum (Untreated RS), Untreated Bovine Serum Albumin (Untreated BSA) and Untreated Egg Albumin (Untreated Ea)

Untreated RS, untreated BSA and untreated Ea were prepared in the same way as untreated GPS.

III) Sensitization Procedure

Forty one male guinea pigs each weighing from 300 to 350 gm were used. The animals were fed with water and solid food CR-I. HN2-GPS compl. and untreated GPS were used as antigen for sensitization. Each antigen was emulsified with a equal volume of Freund's incomplete adjuvant that consisted of 9 vol. of liquid paraffin and 1 vol. of Arlacel A.

0.05 ml of the emulsion was injected intracutaneously on the back of the animals once a week for four weeks. Thirty seven guinea pigs were sensitized with HN2-GPS compl.; 15 of them were used for skin test, 16 for systemic anaphylaxis and 6 for passive cutaneous anaphylaxis. Four guinea pigs were sensitized with untreated GPS.

IV) Skin Test

Skin test was performed 2 or 3 weeks after the last sensitizing injection. Fifteen animals sensitized with HN2-GPS compl. were divided into two groups. The first group comprising 10 animals was tested with HN2-GPS compl.. The second group comprising 5 animals was tested with HN2-RS compl.. In the first group, 0.1 ml of 4 fold dilution of HN2-GPS compl., which contained 0.5 mg of protein, was injected intracutaneously on one side of the belly, and on the other side, untreated GPS was injected as a control agent. After clipping the hair, the injections were done with a short bevel needle for Mantoux's test. In the second group each of the sensitized animals was injected with 0.05 mg of 2 fold dilution of each of HN2-RS compl. and untreated RS, which contained 0.5 mg of protein. A week after the test with HN2-RS compl., the animals of this group were tested again with HN2-GPS compl. and untreated GPS. Depilatory agents were not employed so as to avoid non-specific irritation of the skin. In each animal of the two groups, physiological salt solution was also injected as another control agent. At the same time, non-sensitized guinea pigs were also examined as control animals.

As the third group, 4 control animals which were sensitized with untreated GPS were tested in the same way as in the other two groups. When the sensitized animals responded positively on the test-injection, the site of injection in the skin became erythematous, swollen and elevated. In such a case, the two main diameters of the erythematous lesion were described. From the skin lesion above the panniculus carnosus, preparations for histological examination (hematoxylin-eosin stain) were made 4 hours after the test-injection.

V) Systemic Anaphylaxis

Sixteen guinea pigs sensitized with HN2-GPS compl. were tested for systemic anaphylaxis 2 or 3 weeks after the last sensitizing injection. They were divided into two groups. The first group consisted of 11 animals. Seven of 11 animals were tested by intravenous injection of 1 ml of 4 fold dilution of HN2-GPS compl., i. e. 5 mg of protein, into the superficial vein of the hind leg or the jugular vein. In those animals which did not succumb to the first intravenous test-injection, another test was repeated by injecting 6—15 mg of protein of HN2-GPS compl. 3 weeks later. Four of 11 animals were tested with injection of 15—20 mg of protein of untreated GPS as a control agent. The second group comprising 5 animals was tested with a solution of HN2-RS compl. or HN2-BSA compl., each containing 15—20 mg of protein. As control animals, 4 non-sensitized guinea pigs were tested with HN2-GPS compl..

As the third group, another 4 control animals which were sensitized with untreated GPS and injected thereafter intracutaneously with untreated GPS and HN2-GPS compl.

for skin test, were used again. They were injected intravenously, one week after the skin test, with 10—30 mg of protein of untreated GPS.

After the test-injections, changes of the rectal temperature were measured. When the animals died of typical anaphylaxis, autopsies were done and hematoxylin-eosin preparations of the lung were made for histological examination.

VI) Passive Cutaneous Anaphylaxis (PCA) by OVARY's Method[21)25)]

Three or 4 weeks after the last sensitizing injection, 6 guinea pigs sensitized with HN2-GPS compl. were bled from the jugular artery. The blood from each animal was allowed to clot at 5°C and the serum was collected by decanting and centrifuging. Each serum containing merthiolate in a concentration of 0.01% was kept frozen at −20°C. Thawing and refreezing of serum was avoided as much as possible. The serum was tested with various antigens in vivo as follows.

The serum was diluted 3—10 times with physiological salt solution and the total protein concentration was reduced to be less than 1%. The diluted serum, i. e. antiserum to HN2-GPS compl., was injected intradermally in the abdomen of normal guinea pigs, each weighing 250±50 gm. The hair at the site of injection was simply clipped without any depilatory agents which might cause non-specific irritation of the skin. In performing the intradermal injection, a short bevel needle for Mantoux's test was used and care was taken to deposit the inoculum exactly in the dermal layer. Each normal guinea pig received no more than 3 intradermal injections of 0.1 ml of each of the diluted antisera obtained from three different animals. Six—seven hours after the intradermal injections of the antisera, the animals were challenged with an intravenous injections of various test-antigens mixed with 0.25 ml of 0.5% Evans' blue per 100 gm body weight into jugular vein. The skin where the antisera were injected was examined 30 minutes, 1 hour and at times, 3 hours after the intravenous injection of antigen and Evans' blue.

The animals which received the antisera intradermally were divided into four groups. In the first group, a test-antigen of 10 mg of protein of HN2-GPS compl., i. e. 1 ml of 2 fold dilution of HN2-GPS compl., was injected intravenously to two animals. The other two animals were injected intravenously with the same amount of protein of untreated GPS. In the second group, two and one animals were tested in the same way with 30 mg of protein of HN2-RS compl. and of HN2-Ea compl. respectively. The other three animals were also tested with the same amount of protein of untreated RS or of untreated Ea. In the third group, two animals were injected intravenously with 0.5 ml of a solution of HN2, which was prepared as follows. HN2 was dissolved in physiological salt solution at a concentration of 1 millimol per liter. The solution was used within 5 minutes after it was prepared. In the forth group, two animals were tested with intravenous injection of Evans' blue alone without any test-antigen.

EXPERIMENTAL RESULTS

I) Skin Test

1) Intradermal Test-Injection of HN2-GPS Compl. in Guinea Pigs Sensitized with HN2-GPS Compl. (Table 1)

Ten guinea pigs sensitized with HN2-GPS compl. and tested with HN2-GPS compl.,

No. 1—10, showed eruptions at the site of test-injection. The eruptions were pink or dark pink in color and swollen, elevated and in some cases with petechiae (Photo. 1). These eruptions were 14×12—34×15 mm×mm in size. Histological examination of the skin lesion revealed congestion and marked infiltration of polymorphonuclear leucocytes at the base of the dense dermis above the panniculus carnosus (Photo. 2). These findings indicated that the reactions observed in these animals were not of delayed type but of immediate type. Untreated GPS, a control agent, did not cause any significant reaction.

Table 1 Skin Test in Guinea Pigs Sensitized with HN2-GPS Compl.
(Test-injection of HN2-GPS compl.)

Animal No. \ Test-antigen		HN2-GPS Compl. 0.5 mg 0.1 ml (mm×mm)	Untreated GPS 0.5 mg 0.1 ml (mm×mm)	NaCl 0.1 ml (mm×mm)
Experimental Animals (sensitized)	1	26×16 d. p., swol., el., pet.	3×2 p. p., el.	—
	2	24×14 d. p., swol., el., pet.	—	—
	3	29×14 d. p., swol., el., pet.	—	—
	4	24×16 d. p., swol., el., pet.	5×4 p. p., el.	—
	5	23×12 d. p., swol., el., pet.	3×4 p. p., el.	—
	6	34×15 d. p., swol., el.	3×3 p. p., el.	—
	7	14×12 p., swol., el.	2×2 p. p.	2×2 p. p.
	8	29×16 p., swol.	—	—
	9	26×16 p., swol., el.	—	—
	10	33×21 d. p., swol., el.	2×2 p. p.	—
Control Animals (non-sensitized)	11	—	—	—
	12	—	—	—
	13	—	—	—
	14	—	—	—
	15	2×2 p. p.	—	—
	16	9×8 p.	7×6 p.	—

Abbreviation : p. p. (pale pink), p. (pink), d. p. (dark pink), swol. (sowollen), el. (elevated), pet. (petechiae).

HN2-GPS compl. itself did not have any non-specific irritating effect to the skin as shown in the control animals. For example, the skin preparation obtained from guinea pig No. 16 four hours after test-injection did not show any finding of infiltration of leucocytes.

From these results, it is supposed that HN2 might alter an immunological property of guinea pig serum.

2) Intradermal Test-Injection of HN2-RS Compl. in Guinea Pigs Sensitized with HN2-GPS Compl. (Table 2)

Five guinea pigs sensitized with HN2-GPS compl. were examined with test-injection of HN2-RS compl.. The animals showed eruptions, intensity of which was somewhat weaker than that of the experimental animals in the skin test with HN2-GPS compl.. The reaction against untreated RS, a control agent, was almost of the same grade as that in the control animals. One week after the skin test, the guinea pigs were tested with

Table 2 Skin Test in Guinea Pigs Sensitized with HN2-GPS Compl. (Test-

Animal No. \ Test-antigen		HN2-RS compl. 0.5mg 0.05ml	Untreated RS 0.5mg 0.05ml	NaCl 0.05 ml
		(mm×mm)	(mm×mm)	(mm×mm)
Experimental Animals (sensitized)	56	26×15 p., swol.	2×2 p.	2×2 p.
	57	40×35 p., swol.	?	—
	58	25×20 p. p.	7×7 p. p.	—
	59	23×14 p., swol.	4×3 p.	—
	60	17×14 p.	8×7 p.	—
Control Animals (non-sensitized)	61	4×5 p.	6×7 p.	—
	62	—	—	—
	63	—	8×7 p.	—
	64	3×4 p.	5×4 p.	—

Abbreviation: the same as in Table 1.

HN2-GPS compl. and similar results to those in Table 1 were obtained.

From these results, it is thought that HN2 might take part in determining immunological specificity of a protein to which HN2 is conjugated.

3) Intradermal Test-Injection of Untreated GPS in Guinea Pigs Sensitized with Untreated GPS (Table 3)

Skin test in the third group, i. e. guinea pigs sensitized with untreated GPS, was carried out by injecting untreated GPS and HN2-GPS compl.. There was not any significant difference between the eruptions induced by HN2-GPS compl. and untreated GPS. And cutaneous reactions observed in these animals were definitely slighter than those of the experimental group shown in Table 1.

Table 3 Skin Test in Guinea Pigs Sensitized with Untreated GPS
(Test-injection of untreated GPS)

Animal No. \ Test-antigen	Untreated GPS 0.5mg 0.1ml	HN2-GPS Compl. 0.5mg 0.1ml	NaCl 0.1ml
	(mm×mm)	(mm×mm)	
51	8×7 p., el.	4×4 p., el.	—
52	6×6 p., el.	6×5 p., el.	—
53	9×6 p., el.	5×4 p., el.	—
54	—	—	—

Abbreviation : the same as in Table 1.

Judging from the results shown in Table 1, 2 & 3, it can be said that the reactions induced by the test-injections of HN2-GPS compl. in the experimental group in Table 1 were specific to HN2 treatment.

II) Systemic Anaphylaxis

1) Intravenous Test-Injection of HN2-GPS Compl. in Guinea Pigs Sensitized with HN2-GPS Compl. (Table 4, 5 & 6)

Seven guinea pigs sensitized with HN2-GPS compl., No. 17—23, received intravenous injection of 5 mg of protein of HN2-GPS compl. (Table 4). The animal No. 19 showed signs of distress within a minute after intravenous injection of the antigen. The hair on the head and back of the neck began to ruffle. The animal became restless, and coughed, rubbed its nose and seemed to choke. Soon the animal was gasping for breath and making tremendous inspiratory efforts. The animal defecated, urinated and gave a few convulsive kicks, gasped, and was about to stop breathing. The rectal temperature was markedly dropped. Those findings

injection of HN2-RS compl. or HN2-GPS compl.)

	HN2-GPS compl. 0.5mg 0.05ml	Untreated GPS 0.5mg 0.05ml	NaCl 0.05ml
	(mm×mm)	(mm×mm)	
	48×20 p., swol., el.	12×7 p.	
	22×18 p., swol., el.	10×7 p.	
1 week	22×20 p., swol., el.	6×5 p.	
later	25×16 p., swol., et.	8×7 p.	
	20×18 p., swol., el.	8×7 p.	
	8×7 p., swol., el.	10×8 p., swol, el.	
	6×5 p.	10×7 p.	

are thought to be typical of anaphylactic shock. In the animals No. 17, 20 & 21, moderate symptoms of restlessness, coughs, and respiratory distress were followed by considerable weakness and a drop in rectal temperature. The other animals showed only slight symptoms such as restlessness and coughs.

Three weeks after the test-injection, all 7 animals received another injection of the increased amount of antigen (Table 5). Two of 7 animals were injected with 6 mg of protein of the antigen which did not effect severe anaphylactic shock. The other five

Table 4 Systemic Anaphylaxis in Guinea Pigs Sensitized with HN2-GPS Compl. (Intravenous injection of HN2-GPS compl.)

Animal No.	Amount of test-injection	Symptoms (changes in temperature, °C)	
17	5mg	Coughs, labored breathing	(−0.8)
18	5mg	Coughs, slight symptoms	(−0.6)
19	5mg	Typical anaphylaxis, recovered	(−1.9)
20	5mg	Coughs, labored breathing	(−0.8)
21	5mg	,, ,,	(−1.0)
22	5mg	Coughs, slight symptoms	(−0.6)
23	5mg	Slight symptoms	(−0.6)

Table 5 Systemic Anaphylaxis in Guinea Pigs Sensitized with HN2-GPS Compl. (Intravenous injection* of HN2-GPS compl.)

Animal No.	Amount of test-injection*	Symptoms (changes in temperature, °C)	
17	15mg	Typical anaphylaxis †	6min.
18	15mg	,, ,, †	10min.
19	12mg	,, ,, †	7min.
20	12mg	,, ,, †	4min.
21	10mg	Severe anaphylaxis, recovered	(−3.2)
22	6mg	Coughs, labored breathing	(−0.8)
23	6mg	Slight symptoms	(−0.6)

† Death of animal

* Injected 3 weeks after the 1st intravenous injection of HN2-GPS compl.

animals were injected with 10—15 mg of protein of the antigen and showed typical anaphylaxis. Four of them died within 10 minutes after the intravenous test-injection.

Autopsy of guinea pigs died of typical anaphylaxis revealed that the lung having small hemorrhages on its surface was markedly inflated, and peristalsis of the small intestine was accelerated. Histological investigation of the lung revealed a high degree of emphysema; air vesicle was markedly expanded and the wall of vesicle was thin and blown up (Photo. 3). Some bronchioles were obstructed due to mucous degeneration and stripping of epithelium. Congestion and hemorrhage in the peribronchial area or near the surface of the lung and hyperplasia of lymphatic tissue containing many plasma cells were observed (Photo. 4 & 5).

Table 6 Systemic Anaphylaxis in Guinea Pigs Sensitized with HN2-GPS Compl. (Intravenous injection of untreated GPS)

Animal No	Amount of test-injection	Symptoms (changes in temperature, C)	
24	15mg	No symptoms	(−0.2)
25	15mg	〃 〃	(0)
26	20mg	〃 〃	(−0.1)
27	20mg	〃 〃	(0)

Table 7 Systemic Anaphylaxis in Non-Sensitized Control Guinea Pigs (Intravenous injection of HN2-GPS compl.)

Animal No.	Amount of test-injection	Symptoms (changes in temperature, C)	
28	15mg	No symptoms	(−0.3)
29	15mg	〃	(−0.8)
30	20mg	〃	(−0.1)
31	20mg	〃 〃	(−0.3)

As a control agent of HN2-GPS compl., untreated GPS was injected to 4 guinea pigs sensitized with HN2-GPS compl.. Fifteen—twenty mg of protein of untreated GPS did not cause any distress (Table 6).

Toxicity of HN2-GPS compl. itself was examined by intravenous injection of 15—20 mg of protein of HN2-GPS compl. into 4 non-sensitized control animals. The animals did not show any symptoms such as seen in Table 4 & 5 (Table 7).

2) Intravenous Test-Injection of HN2-RS Compl. or HN2-BSA Compl. in Guinea Pigs Sensitized with HN2-GPS Compl. (Table 8)

In the second group, 5 guinea pigs sensitized with HN2-GPS compl. were examined against HN2-RS compl. and HN2-BSA compl.. No significant symptoms were observed.

Table 8 Systemic Anaphylaxis in Guinea Pigs Sensitized with HN2-GPS Compl. (Intravenous injection of HN2-RS compl. or HN2-BSA compl.)

Animal No.	Amount of test-injection		Symptoms (changes in temperature, C)	
32	HN2-RS Compl.	15mg	No symptoms	(0)
33	〃 〃	30mg	Questionable symptoms	(−0.1)
34	HN2-BSA Compl.	15mg	No symptoms	(−0.4)
35	〃 〃	20mg	Questionable symptoms	( )
36	〃 〃	20mg	No symptoms	(0)

3) Intravenous Test-Injection of Untreated GPS in Guinea Pigs Sensitized with Untreated GPS (Table 9)

As the third group, 4 guinea pigs sensitized with untreated GPS and injected thereafter with untreated GPS and HN2-GPS compl. in order to study the skin test, were tested with intravenous injection of 10—30 mg of protein of untreated GPS, one week after the skin test. The animals did not show any symptoms.

Table 9 Systemic Anaphylaxis in Guinea Pigs Sensitized with Untreated GPS (Intravenous injection of untreated GPS)

Animal No.	Amount of test-injection	Symptoms
51	10mg	No symptoms
52	15mg	,, ,,
53	20mg	,, ,,
54	30mg	,, ,,

Animals were tested 1 week after skin test for HN2-GPS compl. and untreated GPS.

III) Passive Cutaneous Anaphylaxis (PCA) in Guinea Pigs

From the results that the guinea pigs sensitized with HN2-GPS compl. had immediate hypersensitivity to HN2-GPS compl., it is conceived that the animals might have serum antibody to the antigen. Passive cutaneous anaphylaxis in guinea pigs was undertaken to clarify this point.

In general, animals which have been inoculated intradermally with an antiserum in the abdomen show various grade of reaction at the site of inoculation, when an corresponding antigen is given intravenously. In case of positive reaction a more or less regular, circular blue spot develops at the site of inoculation within 30 minutes after intravenous test-injection, which is due to changes in permeability of the capillary wall in consequence of antigen-antibody combination (Photo. 6).

In this experiment, the intensity of the reaction depended to some extent on the amount of intradermally injected antibody and was graded according to the diameter of the blue spot as OVARY and BIER had described[21]: − (no reaction), ± (less than 5 mm), + 5—10 mm), ++ (10—15 mm), +++ (15—20 mm), ++++ (more than 20 mm).

1) Intravenous Challenge with HN2-GPS Compl. (Table 10)

As shown in Table 10, five of 6 antisera caused evidently positive reaction to HN2-

Table 10 PCA in Guinea Pigs Inoculated Intradermally with Antisera to HN2-GPS Compl. (Intravenous challenge of HN2-GPS compl.)

Amount of test-injection \ Antiserum No.		44	45	46	47	48	49	NaCl
HN2-GPS compl. 10mg	30 min.	++	+++	++	++	++	−	−
	1 hr.	++	++++	+++	+++	+++	−	±
Untreated GPS 10mg	30 min.	−	−	−	±	−	−	−
	1 hr.	−	−	−	±	−	−	−

Reaction in each animal was read 30min. and 1 hr. after the test-injection.
Results were graded according to the diameter of the blue spot: − (no reaction), ± (less than 5mm), + (5 — 10mm), ++ (10 — 15mm), +++ (15 — 20mm), ++++ (more than 20mm).

GPS compl. but not to untreated GPS. The results indicated that the guinea pigs sensitized with HN2-GPS compl. had serum antibody to HN2-GPS compl., which could be transferred to the test animals.

2) Intravenous Challenge with HN2-RS Compl. or HN2-Ea Compl. (Table 11 & 12)

Table 11 shows the cross-reaction between HN2-RS compl. and antisera to HN2-GPS compl.. Positive reactions were obtained in the antisera No. 45, 46 & 47, when 30 mg of protein of HN2-RS compl. was challenged. Ten mg of the antigen could not induce clearly positive reaction. The antisera No. 44 & 45 also cross-reacted with 30 mg of protein of HN2-Ea compl. (Table 12), but not with the same amount of untreated RS or untreated Ea.

Table 11 PCA in Guinea Pigs Inoculated Intradermally with Antisera to HN2-RS Compl. (Intravenous challenge of HN2-RS compl.)

Amount of test-injection \ Antiserum No.		44	45	46	47	48	49	NaCl
HN2-RS	30 min.	−	++++	++++	++	−	−	−
compl.	1 hr.	−	++++	++++	++	−	−	−
30mg	3 hrs.	−	++++	++++	++	±	±	±
Untreated	30 min.	−	−	−	−	−	−	−
RS	1 hr.	−	−	−	−	−	−	−
30mg	3 hrs.	−	−	−	−	−	±	−

Reaction in each animal was read 30min., 1 hr. and 3 hr. after the test-injection.
Results were graded in the same way as for Table 10.

Table 12 PCA in Guinea Pigs Inoculated Intradermally with Antisera to HN2-GPS Compl.
(Intravenous challenge of HN2-Ea compl.)

Amount of test-injection \ Antiserum No.		44	45	46
HN2-Ea compl.	30 min.	+++	++	±
30mg	1 hr.	+++	++	±
Untreated Ea	30 min.	−	−	−
30mg	1 hr.	−	−	−

Reaction in each animal was read 30min. and 1hr. after the test-injection.
Results were graded in the same way as in Table 10.

These results indicated that the antibody to HN2-GPS compl. had a reactive group which combined specifically with a structure directly related to HN2.

3) Intravenous Challenge with HN2 (Table 13)

When HN2 was challenged intravenously, the test animals did not show any reaction at the site of inoculation.

4) Intravenous Challenge with Evans' blue (Table 13)

The sera intradermally inoculated were also tested with injection of Evans' blue alone without antigen. No significant reaction was observed.

Table 13 PCA in Guinea Pigs Inoculated Intradermally with Antisera to HN2-GPS Compl. (Intravenous challenge of HN2 solution or Evans' blue alone)

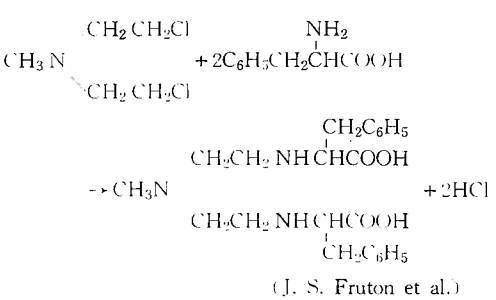
Amount of test-injection	Antiserum No.	44	45	46	47	48	49	NaCl
HN2	30 min.	−	−	−	−	−	−	−
0.7mg/kg	1 hr.	−	−	−	−	±	−	−
Evans'	30 min.	−	−	−	−	−	±	−
blue	1 hr.	−	−	−	−	−	±	−
alone	3 hrs.	−	−	−	−	−	±	−

Reaction in each animal was read 30 min., 1 hr. and 3 hrs. after the test-injection.
Results graded in the same way as in Table 10.

DISCUSSION

LANDSTEINER and his co-workers (1936)[15][16] studied the relationships between immunological specificity and chemical structure by introducing diazo compound into proteins. Sulfanilic acid, after being diazotized, can be coupled mainly to tyrosine groups of a protein such as egg albumin. The antibody obtained by immunizing rabbit with such an azo protein may cross-react with sulfanilic acid coupled to an unrelated protein[16]. Such a simple chemical as sulfanilic acid itself does not give rise to antibody formation when injected. These non-antigenic groups which determine immunological specificity when artificially introduced into proteins have been called "hapten"[13][28]. EISEN, ORRIS & BELMAN (1952)[6] and LANDSTEINER & JACOBS (1936)[17] emphasized that simple chemicals would take a role of hapten when combined irreversibly with tissue constituents, presumably proteins[5][19].

FRUTON, STEIN & BERGMANN (1946)[8] showed that when HN2 was brought into reaction with proteins, their main functional groups which were combined with HN2 might be amino groups, imidazole groups, carboxyl groups, sulfide groups and sulfhydryl groups, and showed that one molecule of HN2 was conjugated with 2 molecules of phenylalanine (Fig. 2). It was showed by ALEXANDER, FOX, STACEY & SMITH (1952)[1] that bovine serum albumin in a 2% (w/v) solution was crosslinked by some alkylating agents such as HN2, bisepoxides and tri(ethyleneimino)triazine (Fig. 3). ISHIDATE (1957)[11] suggested that if Nitrogen Mustard could attack DNA, it might be coupled to amino groups or imido groups on purine or pyrimidine bases of the DNA, which might easily result in intermolecular bridge reaction as shown in Fig. 4. Anyway some of alkylating agents have a function of crosslinking proteins and the combination between the agents and the proteins seems to be irreversible and stable[11][23]. BERENBLUM & WORMALL (1939)[4] showed that rabbit antiserum to mustard gas-treated horse serum reacted with mustard gas-treated



(J. S. Fruton et al.)

Fig. 2

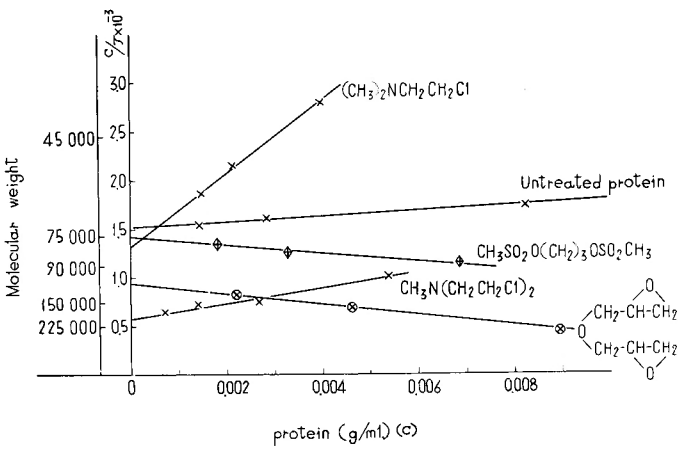


Fig. 3 Molecular weight determination by light scattering of a 2% (w/v) solution of bovine serum albumin after treatment with radiomimetic compounds (2×10^{-2}M). (P. Alexander et al.)

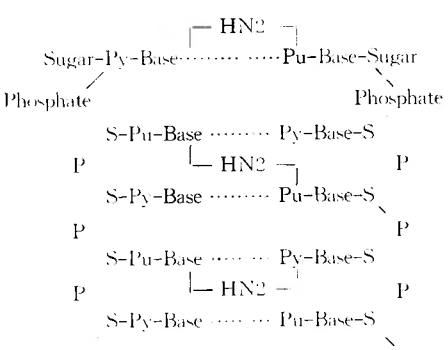


Fig. 4 DNA-Model and Nitrogen Mustard (M. Ishidate)

rabbit serum. These reports mentioned above seem to suggest that some alteration in immunological specificity of protein or nucleoprotein could be induced by those alkylating agents.

In this study, guinea pig serum was coupled to HN2 under a condition of physiological pH and temperature. The use of reagents which might effect non-specific denaturation of the protein, and mechanical damages to the protein was avoided as far as possible. Although HN2-treated guinea pig serum might contain both HN2-guinea pig serum conjugate and unchanged protein, no attempt was made to separate the conjugated protein. The serum treated with HN2 in this way was denominated HN2-guinea pig serum complex. Because the purpose of this study was to determine whether or not HN2 can effect immunologically-detectable changes in proteins under a condition of physiological pH and temperature, the protein was made contact with a fairly large amount of HN2.

Our results obtained by skin test and anaphylaxis indicated that HN2-GPS compl. was immunologically different from untreated GPS. And hypersensitivity observed in the guinea pigs sensitized with HN2-GPS compl. was not of delayed type but of immediate type. Moreover, the results obtained by passive cutaneous anaphylaxis (PCA) showed that the guinea pigs sensitized with HN2-GPS compl. produced serum antibodies to HN2-GPS compl. which cross-reacted with HN2-rabbit serum complex or HN2-egg albumin complex. These results led us to a concept that guinea pig serum might be modified when treated with HN2 and become "foreign" or heterologous to the guinea pigs and that HN2 acted as hapten which determines immunological specificity of this artificially modified antigen. But the antiserum to HN2-GPS compl. did not react with HN2 alone.

Recently Luzzio (1963)[18] reported that human serum γ-globulin after intense in-vitro x-irradiation was distinct immunologically from non-irradiated or heated human serum γ-globulin when examined by quantitative precipitin test.

Although HN2 and its derivatives have been used widely in clinical field, none of

them are really satisfactory in effect. If HN2 and its derivatives could act as a hapten, when coupled to macromolecular substances such as protein or nucleoprotein, these drugs would alter the immunological properties of the protein or nucleoprotein. If so, those altered substances which had been ordinally "self" to the host would become antigenic to the host.

STEWART (1952)[7)24)] reported a case of inoperable uterine myosarcoma. The patient developed, just before the completion of a couse of radiation therapy, a high fever, an urticarial rash and a high eosinophilia, and lost kilos of tumor and ascitic fluid within several days and thereafter showed the hypersensitive reaction repeatedly. The patient survived 10 years. To explain the fortunate course of this patient, STEWART advanced an assumption that some alteration had occurred in the tumor protein and the patient had become sensitized to the altered tumor protein.

Based on the fact[7)14)] that cancer in human beings sometimes regresses, perhaps in consequence of immune response on the part of the host, many trials have been made to detect specific antigen for cancer cells or autoantibodies to cancer. However, it is questionable that the host can produce autoantibodies in the true sense against spontaneous autochthonous tumor. In this regard, there is an idea[27)] in the field of autoimmune deseases that some morbid processes may affect the antigenic structure of some tissue, resulting in stimulating antibody production against the altered antigen. On the other hand, CREECH (1960)[22)] presumed that a chemotherapeutic agent regionally administered might increase antigenicity of tumor and enhance development of autoantibodies.

Recently, NAGAMATSU[20)] vaccinated dd-strain mice subcutaneously with Ehrlich ascites carcinoma which were treated with Mitomycin C in vivo. A couple of weeks after the vaccination, 10^6 cells of Ehrlich ascites carcinoma were transplanted intraperitoneally to the vaccinated mice and two days later, 1 mg of Mitomycin C per kg body weight was injected intraperitoneally to the mice. Although the amount of Mitomycin C was not enough to inhibit growth of the tumor when administered alone to non-vaccinated mice, it could inhibit growth of the tumor in nearly 100% of the vaccinated mice. In his experiment, some substances in the tumor tissue, immunological property of which might have been altered by the treatment with Mitomycin C, must have take an important role to inhibit the tumor growth.

In order to examine the possibility that this kind of modification of antigenic property can be utilized in the field of experimental treatment of cancer, further study has been undertaken using spontaneous mammary cancer of mice.

SUMMARY AND CONCLUSION

The investigation was undertaken to elucidate whether HN2 coupled to proteins would effect immunologically detectable changes in the proteins. Guinea pig serum was treated with HN2 to make HN2-guinea pig serum complex (HN2-GPS compl.). The guinea pigs sensitized with the complex were tested by skin test, systemic anaphylaxis and passive cutaneous anaphylaxis. The results were as follows.

1) In skin test, guinea pigs sensitized with HN2-GPS compl. showed immediate type of hypersensitivity to HN2-GPS compl. and at the same time cross-reacted to HN2-rabbit

serum complex, while they did not react to untreated GPS.

2) Guinea pigs sensitized with HN2-GPS compl. showed typical systemic anaphylaxis to HN2-GPS compl. and the histological preparations of the lungs showed characteristic findings of anaphylactic shock. The animals did not respond on intravenous injection of HN2-rabbit serum complex or HN2-bovine serum albumin complex.

3) The serum from guinea pigs sensitized with HN2-GPS compl. was tested by passive cutaneous anaphylactic reaction and it was indicated that the guinea pigs produced serum antibodies to HN2-GPS compl.. These antibodies cross-reacted to HN2-rabbit serum complex and HN2-egg albumin complex.

4) From these results, it can be said that HN2 might conjugate with proteins, perhaps by means of crosslinking, and confer new immunological specificity on the proteins. The possibility that this kind of modification of antigenic property of such substance as protein or nucleoprotein in tumor tissue might be utilized in the field of experimental treatment of cancer was discussed.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Assistant Professor Dr. IKUZO YOKOYAMA for his many valuable suggestions and helpful discussions throughout the experiment, and to Dr. KANAFU TABYI, Professor of Department of Microbiology of Kyoto University, who critically reviewed the manuscript.

The author is also very grateful to Dr. KIRO SHIMAMOTO, Professor of Department of Pharmacology of Kyoto University, for his helpful discussions on the data, and to Dr. MASAHISA KYOGOKU, instructor of Department of Pathology of Kyoto University, for his kind guidances in histological investigation.

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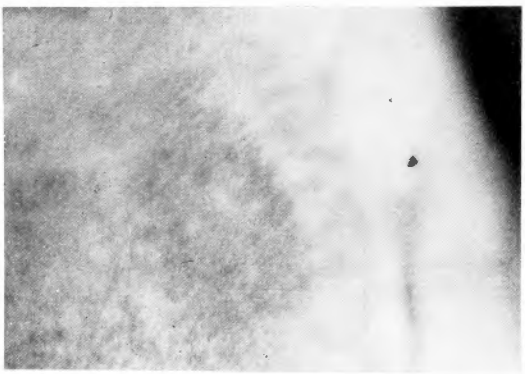

Photo. 1 Cutaneous reaction in guinea pig sensitized with HN2-GPS compl., 4 hrs. after intracutaneous test-injection of HN2-GPS compl..

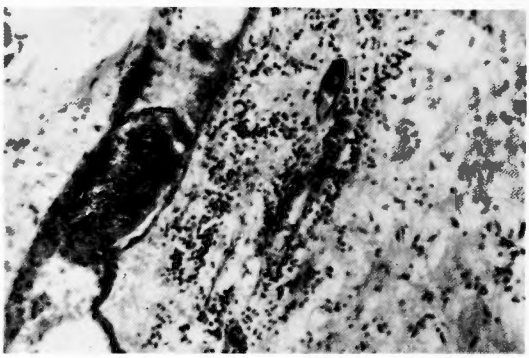

Photo. 2 Diffuse infiltration of polymorphs and congestion in skin lesion from guinea pig sensitized with HN2-GPS compl., 4 hrs. after intracutaneous test-injection of HN2-GPS compl..
×50

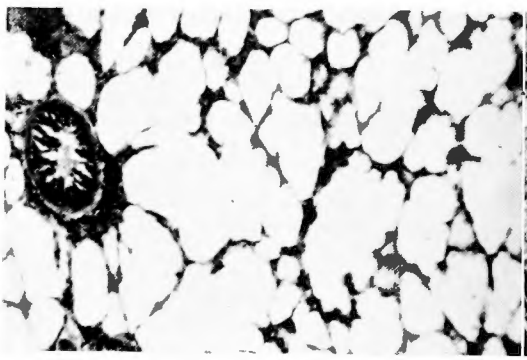

Photo. 3 Emphysema in lung from guinea pig which died of typical systemic anaphylaxis.
×50

Photo. 4 Hemorrhage and hyperplasia of lymphatic tissue in peribronchial area, from guinea pig which died of typical systemic anaphylaxis.
×50

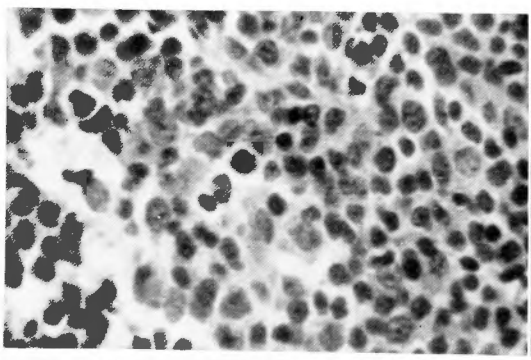

Photo. 5 Lymphatic tissue containing many plasma cells, from guinea pig which died of typical systemic anaphylaxis. ×200

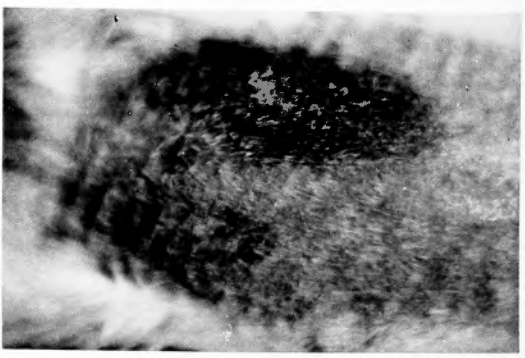

Photo. 6 Blue dye accumulation in guinea pig at site of inoculation of guinea pig antiserum to HN2-GPS compl., as a result of intravenous challenge of HN2-GPS compl. mixed with Evans' blue 6 hrs. after the intradermal inoculation of the antiserum.

和文抄録

Nitrogen Mustard 処理モルモット血清のモルモットに対する抗原性について

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朝隈六郎

P.Alexander 等(1959)はニシン(鯡)の精子のnucleoprotamine に nitrogen mustards, epoxides 或いは ethyleneimines 等のアルキル化剤が作用すると，相異なるDNA分子間の結合，即ち，intermolecular crosslinking の生起する事を示した．一方，K.Landsteiner等(1936)は，アトキシールを初め種々の化学基を蛋白に結合させ，これによつて，動物を免疫すると，導入された化学基に特異的に反応する抗体が生ずる事を発見し，この化学基は抗原の構造を変える事により，免疫学的特異性を支配するものである事を明らかにした．

そこで，nitrogen mustard (HN2) が人工的に，蛋白に結合された場合，HN2 は，その蛋白の免疫学的特異性を変えるのではないかと考えられるので以下の実験を行つた．

蛋白濃度2％のモルモット血清に，HN2 を 2×10^{-2}M の濃度に加え，24時間，37 C，pH 7.5 に保つて，HN2-モルモット血清複合物 (HN2-GPS compl.) を作製した．これを背部皮内に注射することによつて，感作されたモルモットについて，skin test 或いは systemic anaphylaxis にて，HN2-GPS compl., 無処理モルモット血清或いは HN2-家兎血清複合物 (HN2-RS compl.) に対する過敏性を検した．次いで，HN2-GPS compl. 感作モルモットの血清を，正常モルモットの皮内に移して，passive cutaneous anaphylaxis (PCA) を行い，HN2-GPS compl.に対する抗体の有無及び HN2-RS compl. 或いはHN2-卵白アルブミン複合物 (HN2-Ea compl.) に対する交叉反応性を調べ，以下の成績を得た．

1) HN2-GPS compl.感作モルモットは HN2-GPS compl. に対し，即時型の皮膚過敏性を示し，反応は試験注射後，4時間目に観察し，発赤部の皮膚は組織学的に，多核白血球の浸潤，充血を示した．無処理モルモット血清に対しては，意義ある反応を示さなかつた．

2) HN2-GPS compl.感作モルモットは，蛋白量10～15mgのHN2-GPS compl.の静注に対してtypical systemic anaphylaxisの症状を示し，shock死したモルモットの肺は，肺気腫，形質細胞に富むリンパ組織の増殖等の組織像を示した．蛋白量 15～20mg の HN2-RS compl.の静注に対しては反応を示さなかつた．

3) PCA法により，HN2-GPS compl.感作モルモットより得た血清は，HN2-GPS compl. と反応し，且つHN2-RS compl. 及びHN2-Ea compl. に対して交叉反応性を示す事を認めた．

4) これらの結果から，HN2 は，恐らく蛋白分子間に橋状に結合して，その蛋白に新しい免疫学的特異性を与え，生じた抗原の決定基 (determinant group) に，HN2 が関与するものと考えられる．この種の抗原性の修飾が，癌の実験的治療に応用し得るかの可能性についても，考察を加えた．