EXPERIMENTAL NONTHROMBOCYTOPENIC VASCULAR PURPURA: A REVIEW OF THE JAPANESE LITERATURE, WITH PRELIMINARY CONFIRMATORY REPORT

By William G. Clark, Ph.D., and Edith Jacobs, B.A.

Katsura,1,2 Ohkubo,2 Okano and Kawakami4 and Hiramatsu5 have published papers in the Japanese language* on experimental nonthrombocytopenic vascular purpura induced by anti-blood vessel endothelium serum. Bedson and Johnston6 produced an antiserum against mononuclear "endothelial" type leukocytes obtained from peritoneal exudates induced by old tuberculin injections, but the antiserum was not purpurigenic.

The Japanese investigators obtained their antigenic material by scraping the endothelium from the lining of the aortas of guinea pigs, and produced the antiserum by injecting this material intravenously in rabbits. Hiramatsu, for example, injected increasing amounts of antigen every few days for a month from as many as 75 guinea pigs into a single rabbit.

Immunologic studies in vitro were made by Katsura, Ohkuba and Hiramatsu, with essentially similar results. The antiserum agglutinated guinea pig platelets, erythrocytes, leukocytes and vascular endothelial cells. It lysed erythrocytes only at high serum concentration (1:10); was not lytic to leukocytes or platelets; and according to Katsura, apparently markedly damaged vascular endothelial cells (swelling, lysis), although Hiramatsu did not confirm this. Normal rabbit serum had none of these effects.

Complement fixation tests performed by Katsura between the antiserum and vascular endothelial cells were positive, and both vascular as well as peritoneal exudate endothelial cells gave positive fixation. When the antiserum was injected into homologous animals, a generalized hemorrhagic purpura occurred, especially in the skin, lungs, gastrointestinal tract, mesenteries and diaphragm. No change was seen in the number of circulating platelets, and Okano and Kawakami observed no alteration of the platelet count in the spleen or lungs. Katsura found that intraperitoneal injection gave the most severe purpura, followed in order by the intravenous and subcutaneous routes. Intra-arterial injection gave a poor reaction.

Hiramatsu found that the purpura was not as severe as that induced by anti-

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*Translations of these were obtained at the author's instigation through the interest and cooperation of General Crawford F. Sams, and Dr. Herbert Volk, General Headquarters, Supreme Command for the Allied Powers, Public Health and Welfare Section, Tokyo; and Mr. Chobei Takeda XII, Takeda Pharmaceutical Industries, Ltd., Osaka, Japan. The paper of Hiramatsu briefly reviews the other Japanese papers on the subject and may be obtained as a photostat (see bibliography). The authors will be glad to supply abstracts of the other translations upon request.
blood platelet serum. Thrombus formation was common, followed by localized areas of ischemia and necrosis and fatty infiltration especially in the liver but also in the spleen and kidney.

The antiserum caused a slight increase in circulating erythrocytes, an occasional transient leukocytosis, and an occasional alteration of bleeding time; but no change in "absolute clotting time," coagulation time or clot retraction time.

Katsura found that when subpurpurigenic amounts of anti-platelet serum (which still produced thrombopenia) and anti-endothelium serum (which still damaged endothelial cells in vivo) were given simultaneously, synergism occurred, and hemorrhagic purpura occurred.

Katsura also found that antiplatelet serum agglutinated endothelial cells in vitro and damaged them (swelling, sloughing) in vivo. With endothelial cells as antigen and anti-platelet serum as antibody, the complement fixation test was positive.

When anti-platelet serum was absorbed by platelets, he found a marked decrease in agglutination of platelets by the absorbed serum in vitro, but when it was absorbed by endothelial cells, platelet agglutination remained unaltered and thrombocytopenic purpura was induced as usual in vivo.

So far as can be determined from the translations available, no further cross absorption studies were made by these workers in order to elucidate the immunologic specificity of this particular cytotoxic antiserum, such as has been made by many other investigators in the case of antiserum of platelets, various types of leukocytes, erythrocytes and the stroma and plasma extracts of these blood cells; and various organ antiserum.

The Japanese authors believed that the purpura induced by injections of the anti-vascular endothelium was a result of diapedesis of blood cells and enhanced capillary permeability and filtration due to specific lesions of the vascular endothelium. In agreement with various occidental investigators such as Nolf, Zung and Govaerts, Roskam, Bedson, Elliott and Whipple, Quick, et al. and others, the Japanese workers believed that the thrombocytopenic purpura caused by anti-platelet serum is due to a combination of the damaging effect of almost any type of cytotoxin on vascular endothelium together with thrombocytopenia, capillary dilatation and other factors which have been discussed by Tocantins.

In the course of investigations designed to discover possible treatments and drugs which might combat various diatheses, an opportunity was had to repeat and extend some of the Japanese observations. Accordingly, in addition to bringing the attention of interested occidental investigators to the Japanese papers briefly reviewed above, it was thought worthwhile to report this work in preliminary form at the present time.

METHODS

Early in the work it was found that the labor involved in trying to obtain vascular endothelium in quantity from the blood vessels of animals as small as guinea pigs and rabbits was extremely tedious. Hence attempts were first made
to produce nonthrombocytopenic vascular purpura with antiserum prepared against other possible rich sources of vascular endothelium.

In all cases, rabbits were used to produce antibodies. Antisera were made against guinea pig whole blood vessels, aqueous extract of homogenized guinea pig and mouse adipose tissue, guinea pig and mouse spleen, bone marrow, and 'antireticulo-endothelial cytotoxic serum,'* guinea pig and mouse platelets, guinea pig choroid plexus and guinea pig urinary bladder (smooth muscle).

The anti-bladder serum was made in the hope that a smooth muscle antiserum could be made and used to absorb out this particular component possibly present in the whole blood vessel antiserum, since it was felt that the latter might be polyvalent with respect to interfering cross reacting antibodies.

Adipose tissue was used because it is rich in nonmuscular small arteries, arterioles and capillaries, and choroid plexi because of their rich capillary supply. Unfortunately, the plexi also contain even more epithelial cells than they do endothelial.

In most cases the antigenic material was frozen with dry ice and thoroughly homogenized in a refrigerated Waring Blendor, then absorbed on aluminum hydroxide gel, by methods described elsewhere, and injected intramuscularly into multiple sites into the rabbits. After a suitable period of time, usually fourteen days, heart blood was obtained and tested against freshly prepared antigen by complement fixation and precipitin tests. Following this, 5 to 10 cc. Upjohn adrenal cortical extract [and in two cases, Upjohn 'Lipoadrenal' extract, rich in 'carbohydrate factor'], were given subcutaneously, and six to eight hours later the animals were exsanguinated, by heart puncture, for serum. This second serum was retested to see if the titers had increased by an anamnestic response to the adrenal cortical hormone, as claimed by Chase, White and Dougherty. At the same time, the antisera were injected by various routes into normal animals of homologous species, including intraperitoneal, intravenous, subcutaneous and intracutaneous, to see if purpuric reactions occurred. In some cases, intracutaneous injections of serial dilutions of some of the antisera were made in accordance with the method of Tocantins to ascertain the purpurigenic titer in vivo, if indeed any purpuric activity was present.

When purpuric responses occurred, platelet counts were made in freshly drawn heart blood by the direct method of Rees and Ecker, as modified by Tocantins, using a nonsilvered Sharp and Smith counting chamber.

In some experiments determinations were made of bleeding time, by a modification of the method of Roskam and Ungar, as well as erythrocyte counts, hematocrit, plasma protein by the falling drop method and rectal temperatures; and in all cases careful observations were made of clinical symptoms and vascular and hemorrhagic reactions seen at autopsy.

Details of the preparation, titration and results in vivo of all the antisera produced will not be described because of space limitations and because the results were negative in vivo.†

* The latter three sera were generously supplied by Dr. Reuben Strauss, then at Cedars of Lebanon Hospital, Los Angeles.
† The authors will be glad to supply these data upon request.
The results of the studies on production of the spleen and platelet antisera are included because they illustrate the superiority of the use of a single multiple injection of aluminum hydroxide gel-absorbed antigen over repeated intravenous administration and because they illustrate to some extent the anamnestic response to adrenal cortical hormone. These two antisera, like toxic doses of "antireticuloendothelialcytotoxic serum," anti-bone marrow, and Forssman heterophil antibody (anti-sheep erythrocyte serum), produce hemorrhagic thrombocytopenic purpura when properly administered, so other than to illustrate the points described, are not pertinent to the present report.

Only the preparation of the anti-vascular endothelium serum will be dealt with in any detail.

Large, healthy mongrel dogs of 75 to 100 pounds body weight were obtained immediately after asphyxiation with carbon monoxide at the city pound. The blood vessels were rinsed free of blood with saline while intact in situ. Using clean but not sterile technic,24 as much as possible was removed of the dorsal ascending and descending aortas and the vena cava. The vessels were slit open longitudinally, pinned open on cork and gently washed with saline and dried lightly with lens paper. The endothelial lining then was scraped off lightly with a spatula and suspended in 5 cc. saline, this mixture being shaken with an equal volume of freshly prepared, sterile aluminum hydroxide gel.2124*

The gel-adsorbed antigen was injected into a 4 pound New Zealand rabbit, 2 cc.† intramuscularly into five different sites. Antigen from 3 different dogs was injected into this same rabbit on three successive days. Fifteen days after the first injection, a heart blood sample was obtained for precipitin and complement fixation tests, and the rabbit was injected subcutaneously with 10 cc. of Upjohn adrenal cortical extract. The rabbit was exsanguinated for serum by heart puncture eight hours later, and the serum retested immunologically using fresh antigen obtained from 2 more dogs.

Five cc. of the antiserum were then injected subcutaneously into two 20 pound healthy, mongrel dogs with light-colored fur and skin. In one of these, platelet, erythrocyte and leukocyte counts and differentials were made before and twenty-four hours after the serum injection at which time both dogs were sacrificed and autopsied. In a third dog, an equivalent amount of normal rabbit serum was injected and blood studies conducted before and twenty-four hours later.

* Prepared as follows:
(1) 10 Gm. \(\text{Al}_3(\text{SO}_4)_2 + (\text{NH}_4)_2\text{SO}_4\cdot 13\text{H}_2\text{O}\) in one liter of \(\text{H}_2\text{O}\) (= 1 per cent)
(2) 2.38 cc. of \(2\text{M NH}_4\text{OH}\) in 100 cc. \(\text{H}_2\text{O}\) (= 1 per cent)

A slight excess of (2) is added to (1) at room temperature in a tall cylinder. The resulting precipitate is allowed to settle, and the supernatant siphoned off. The precipitate is centrifuged, resuspended in \(\text{H}_2\text{O}\), recenterfuged, etc., until the supernatant is \(\text{NH}_4\)-free by Nessler's test. The final precipitate should be of the consistency of thick cream and can be autoclaved. Commercial, dry preparations of aluminum hydroxide gels are unsatisfactory as protein adsorbents. The antigen suspension is treated by shaking with an equal volume of this gel. All protein should be adsorbed, as tested by a Biuret or Million test on the supernatant. If not, not enough gel was used. Some bacterial contamination is permissible.24

† More than 2 cc. may cause pressure necrosis.
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RESULTS

Table 1 briefly summarizes the results. It is apparent that the aluminum gel, single injection method gives higher titers than multiple, intravenous injections. In all cases tried on a positive serum, adrenal cortical extract, but not "Lipo-adrenal" extract, produced a slight anamnestic antibody response, and a marked one in the case of the anti-blood vessel serum.

A good complement fixation titer in vitro was not obtained for the choroid plexus antiserum, although it was positive. No reaction could be obtained at all

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<td>G. pig anti-choroid plexus</td>
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<td>Dog anti-blood vessel endothelium</td>
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* Reactions of 4+ recorded only.
† For descriptions, see text.
‡ Agglutinin tests negative.

for the anti-endothelium serum. This possibly was due to too much dilution of the antigen, but no opportunity has been had to repeat the experiment at this time. The Japanese workers obtained positive tests, although of low titers (Hiramatsu, for example, obtained only a + agglutination at 1:20 serum dilution, ± at 1:120, and — at 1:160). In future experiments it is planned to express the titers on the basis of Kjehldahl nitrogen, in order to quantitate the results.

The choroid plexus antiserum produced a massive edema in the entire cutaneous and subcutaneous regions of the guinea pigs, particularly on the abdomen, even though injected on the back. Normal rabbit serum did not do this. There were, however, no purpuric hemorrhages, no change in the blood picture, hematocrit, plasma protein or bleeding time; there was some elevation of rectal temperature.

Fig. 1 (p. 325).—Gross hemorrhagic response in dog injected with vascular endothelium antiserum.
The anti-adipose serum produced serum sickness, with fever and flushing, but no purpuric symptoms despite good titers in vitro.

The anti-whole blood vessel serum, although it too gave a good titer in vitro produced no symptoms in vivo. Attempts to absorb out a possible masking smooth muscle component by anti-bladder serum had no effect either in vitro or in vivo, which is not surprising in view of the findings of Fleisher and Arnstein\textsuperscript{32} who found in cross absorption and complement fixation experiments with various tissue antisera that anti-muscle serum had the lowest specificity comparatively.

The dogs injected with the vascular endothelium antiserum had a slight fever which subsided in ten hours. They had a marked hemorrhagic purpura, which spread from the site of injection (shoulder) throughout the entire subcutaneous skin and muscular areas of the dorsal side of the body. Internally there were ecchymoses in the gastrointestinal tract, and the spleens were enormously enlarged, engorged, and dark. Figure 1 shows the gross hemorrhagic response in one of the dogs.

There were no significant changes in the platelet, leukocyte or erythrocyte counts nor the differentials. There was no evidence of hematuria.

Normal rabbit serum produced no such reactions in the third dog.

No further studies have been made since these preliminary experiments, but are contemplated.

**DISCUSSION**

This type of purpura\textsuperscript{*} may be a useful tool for studying the immuno-hematologic aspects of various purpuras. If used in conjunction with antiplatelet serum and plasma from patients with various hemorrhagic disorders, human anti-vascular endothelium serum might be interesting for studying the differentiation of various purpuras, along the same lines that have been utilized by Steinberg and Martin\textsuperscript{33} by leukagglutination studies in various disorders of the lymphatic system, and by Morawitz and Brugsch\textsuperscript{35} by platelet agglutination studies in various thrombopathic disorders. Antiserum has been made against human platelets\textsuperscript{36} and it should not be too much of a chore to make human anti-vascular endothelium serum.

Whether or not 'stimulating' doses of anti-endothelial cytotoxic serum would have therapeutic effects is doubtful, in view of the equivocal results with 'anti-reticuloendothelial' cytotoxic sera which have been reported.

In the search for anti-hemorrhagic measures and agents for the treatment of various hemorrhagic diatheses, antiplatelet serum already has demonstrated its worth,\textsuperscript{2} Hiramatsu\textsuperscript{5} claimed that hesperidin-4'-ethylcarbonate (soluble

\textsuperscript{*} In a personal discussion with Dr. L. M. Tocantins at Jefferson Medical College in Philadelphia, it was learned that after seeing a translation of Hiramatsu's paper made available to him by the authors, he was motivated to repeat an earlier unsuccessful attempt to produce nonthrombocytopenic purpura with anti-vascular endothelium serum. In his earlier attempt he employed insufficient amounts of antigen. Accordingly antigen from blood vessels of a very large number of guinea pigs was injected periodically into rabbits, and an active serum was obtained, although it is not known to us whether he made immunologic studies. When injected into guinea pigs, a marked nonthrombocytopenic purpura resulted. Histologic studies revealed generalized dissolution of vascular endothelium, resembling that seen in certain instances of Schönlein-Henoch's purpura. It is to be hoped that these studies will be reported in detail, since we have as yet had no opportunity to make histologic studies.
hesperidin, "vitamin P") exerted more anti-hemorrhagic effect in experimental nonthrombocytopenic vascular purpura than in thrombocytopenic purpura. It is conceivable that such approaches will be useful in elucidating various treatments or drugs which may have specific effects in hemorrhagic diatheses such as radiation disease, Schönlein-Henoch’s purpura, Werlhof’s purpura, and other hemorrhagic disorders.

SUMMARY

Antisera were produced against guinea pig and mouse blood platelets, guinea pig and mouse spleen, mouse bone marrow, spleen, marrow plus spleen ("antireticulocytotoxic" serum), aqueous fraction of guinea pig adipose tissue rich in nonmuscular blood vessels, guinea pig choroid plexi rich in capillaries, whole guinea pig blood vessels, guinea pig smooth muscle, whole guinea pig blood vessel antiserum absorbed with guinea pig smooth muscle, and dog vascular endothelium.

In all cases except the last named there were positive agglutinin and/or complement fixation titers, which were slightly enhanced anamnestically by whole adrenal cortical extract, but not by "Lipoadrenal" extract presumably high in "carbohydrate" factor.

In no case was there a nonthrombocytopenic vascular purpura produced in vivo except by the anti-vascular endothelium serum.

These preliminary observations are considered to confirm and extend observations made in previous reports in Japanese-language periodicals, which are reviewed.

REFERENCES

2 ———: Untersuchungen über die experimentelle Purpura hemorrhagica beim Meerschweinchen; Purpura hemorrhagica verursacht durch Hämangioendothelitoxin. Tr. Japan Path. Soc. 24: 154, 1924.
7 Nolf, P. Quoted by Bedson, S. P.: See reference 10 below; and by Elliott and Whipple. See reference 11 below.
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