LOCALIZATION OF ANTIBODIES
IN SPONTANEOUS AND INDUCED TUMORS

Final Report

David Pressman, Ph.D.

Health Research, Inc.
Buffalo, New York

November 1, 1960 - October 31, 1972

PREPARED FOR THE U. S. ATOMIC ENERGY COMMISSION
UNDER CONTRACT NO. AT(11-1)-3141

Note: This project is being continued without additional support

NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

MASTER

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

TABLE OF CONTENTS

	Page
<u>Project Abstract</u>	3
<u>Scientific Background</u>	4
<u>Research Accomplishments</u>	
Antibodies to Plasma Cell Tumors	7
Antibodies to Acetaminofluorene Induced Tumors	9
Cross Reactions of Antigens in Hepatomas	13
Immunoglobulin (IgG) Associated with Microsomes from Autochthonous Hepatomas and Normal Livers of Rats	13
Localization of Antibodies to Cultured Cells	17
Localization of Normal Serum Globulin Components	17
Distribution of Histocompatibility Antigens in Tissues	18
Radioimmunoassay of Specific Antigens in Tissue Sections	20
Localization Properties of Anti-Moloney Lymphoma Antibodies	21
Immunosuppressive Activity of Rabbit Antisera Directed Against Mouse Lymphocytic Leukemia L1210	22
The Localization of Mouse Alloantibodies of Known Specificities	23
Computation of Results of Paired and Triad Label Techniques	25
<u>Evaluation of Progress</u>	26
<u>Bibliography</u>	27
<u>State of Knowledge In This Area</u>	31

Project Abstract

Radioactive anti-tumor and anti-normal tissue antibodies are being used to determine the antigenic composition of tumors and differences between tumors and normal tissues. A primary aim is to develop radio-labeled antibodies capable of localizing in high enough concentration in tumors so that they can be used as a diagnostic means of determining the location of tumors and perhaps used therapeutically as a means of carrying large amounts of radioactivity to the tumor.

ALTHOUGH SUPPORT IS BEING TERMINATED BY THE AEC, THIS PROJECT IS BEING CONTINUED IN OUR LABORATORY.

Scientific Background

That anti-tissue antibodies can be labeled with radioactive iodine and shown to localize in the tissue against which the antibody was prepared was first shown by Pressman and Keighley in 1948 (1). Since then, other radioactive labels have been used and several tissues have been studied. Radiolabeled antibodies were first shown to be fixed in a tumor, the transplanted Wagner osteogenic sarcoma in mice, by Pressman and Kornfeld in 1953 (2). The use of the radiolabeled antibodies for fixation in tumors and in normal tissues is currently being pursued in several laboratories throughout the world, as well as our own.

Most recently we have been studying the plasma cell tumors in mice and have been able to show antigenic differences between individual tumors as described in the progress report. Previously we studied the acetaminofluorene induced hepatoma of rats (3). It was possible to prepare antibodies which localized to an appreciable amount in the tumor, although there were extensive cross reactions, in that antibodies formed against the hepatoma localized in other tissues also and antibody against normal liver localized in hepatoma (4, 5). It was further possible to separate out antibodies showing a preferential localization in the tumor (6). The tumor was fractionated into the various cytoplasmic components and the greatest specificity has been obtained with the microsome fraction. Although there are apparently common constituents between the individually induced tumors, there are also individual differences which can be detected immunologically (7).

Studies have also been carried on in our laboratory of the localization of antibodies to other tumors, for example the Moloney lymphoma in mice (8), and on the localization of antibodies directed various normal tissues (9, 10, 11) and against various histocompatibility antigens (12) and alloantigens (13, 14).

References

1. Pressman, D. and Keighly, G. The Zone of Activity of Antibodies as Determined by the Use of Radioactive Tracers; the Zone of Activity of Nephrotoxic Antikidney Serum, *J. Immunol.* 59: 141, 1948.
2. Pressman, D. and Korngold, L. The in vivo Localization of Anti-Wagner-Osteogenic Sarcoma Antibodies, *Cancer* 6: 619, 1953.
3. Day, E.D., Planinsek, J. and Pressman, D. Localization of Radioiodinated Antibodies in Rats Bearing Tumors Induced by N-2-Fluorenylacetamide, *J. Natl. Can. Inst.* 25: 787, 1960.
4. Isojima, S., Bernecky, J., Planinsek, J., Yagi, Y. and Pressman, D. Differences Between Antibodies Against the Dense Sediment and Microsome Fractions of the N-2-Fluorenylacetamide-Induced Rat Hepatoma, *Cancer Res.* 25: 968, 1965.
5. Isojima, S., Yagi, Y., Planinsek, J. and Pressman, D. Purification of Localizing Antibodies Formed Against Microsomes of the N-2-Fluorenylacetamide-Induced Rat Hepatoma, *Cancer Res.* 25: 962, 1965.
6. Isojima, S., Planinsek, J., Yagi, Y. and Pressman, D. Differences between the Microsomes of Normal Rat Liver and of N-2-Fluorenylacetamide-induced Rat Hepatoma as Determined by the Paired Label Antibody Technic, *Cancer Res.* 26: 1527, 1966.

7. Isojima, S., Yagi, Y. and Pressman, D. Antigens Common to Rat Hepatomas Induced with 2-Acetylaminofluorene. *Cancer Res.* 29: 140-144, 1969.
8. Witz, I., Klein, G. and Pressman, D. Moloney Lymphoma Antibodies from Mice; Localization in Spleens of Moloney Lymphoma Bearing Mice. *P.S.E.B.M.* 130: 1102-1105, 1969.
9. Tsuzuku, O., Yagi, Y. and Pressman, D. Preparative Purification of Lung-Localizing Rabbit Anti-Rat Lung Antibodies In Vitro. *J. Immunol.*, 98: 1004, 1967.
10. Tsuzuku, O., Yagi, Y. and Pressman, D. Isolation of Rat Lung Antigens Capable of Neutralizing Localizing Activity of Anti-Lung Antibodies. *J. Immunol.*, 98: 1053, 1967.
11. Tsuzuku, O., Yagi, Y. and Pressman, D. Highly Specific Lung Localizing Antibodies. *J. Immunol.* 99: 1, 1967.
12. Witz, I., Yagi, Y. and Pressman, D. Availability of Isoantigens to Circulating Antibodies. *J. Immunol.* 101: 217-223, 1968.
13. Witz, I., Kaliss, N. and Pressman, D. Isoantigens Available to Circulating Antibodies. *J. Immunol.* 102: 283-287, 1969.
14. Witz, I., Yagi, Y. and Pressman, D. Brain Localizing Activity of Hetero- and Isoimmune Sera Directed Against Lymphocytic Cells. *P.S.E.B.M.* 130: 928-930, 1969.

RESEARCH ACCOMPLISHMENTS

Antibodies to Plasma Cell Tumors.- Antisera prepared against lymphocytes and against macrophages have received a great deal of attention in the past few years because of their immunosuppressive activity. However, there has been little work on antibodies against the other important component of immune-reactive cells, plasma cells. Because of the difficulty in collecting normal plasma cells, we have started to study the in vivo and in vitro properties of antibodies against neoplastic plasma cells (myelomas) which were originally induced in Balb/c mice with mineral oil by M. Potter. Antibodies were prepared in rabbits against insoluble components of 6 individual tumors (MOPC-21, MOPC-104E, MOPC-195, RPC-5, MPC-1, Adj-PC-22A) and of a mixture of spleen cells and lymph node cells.

The localization of i.v.-injected rabbit anti-plasma cell antibody in normal and tumor-bearing mice was studied as follows. ^{125}I -Labeled γ -globulin from antiserum against plasma cell tumors and ^{131}I -labeled normal rabbit γ -globulin were mixed and the mixture was injected into animals. After 18 hrs, the animals were perfused and the radioactivity of excised organs was determined (paired label antibody technique). There was very significant localization of antibody in spleen and liver: the ratio of ^{125}I -antiserum globulin to ^{131}I -normal globulin fixed was 4-13 for spleen and 3-4 for liver. Slight localization was observed in lung and kidney and no localization was observed in thymus, lymph node and heart. It was difficult to determine specific localization in solid tumors. No clear-cut specific localization was seen because of the high background. The localization in the spleen could be largely removed by absorbing the antiserum globulin with spleen cells indicating the specificity of localization. Studies are in progress with antibody

preparations specifically purified by the use of insoluble sediments from tumors and other cells.

The antibody activities of these sera were also assayed in vitro by fluoro-chromasia cytotoxicity tests with rabbit complement using homologous and heterologous cells as targets. Anti-tumor sera (absorbed with Balb/c serum and Balb/c erythrocytes) exhibited a strong cytotoxic action against tumor cells of all the lines indicating the presence of common antigens in these tumors. The cytotoxic titers (maximum dilutions of antiserum causing lysis over 99% of target cells) were always highest for the tumor cells used for the immunization, but fell within a factor of 4 for tumor cells of other lines. The cytotoxic titers against spleen and lymph node cells, however, were much lower and were in the range of 1/30 to 1/60 of those for the homologous tumor. The cytotoxicity of anti-tumor sera against spleen and lymph node cells could be completely removed by absorption with spleen or lymph node cells without affecting the cytotoxic activity against tumor cells.

On the contrary, antisera against spleen and lymph node cells showed much higher cytotoxic titers against spleen or lymph node cells than tumor cells and the activity against tumor cells could be completely removed by absorption of antisera with tumor cells without affecting the cytotoxic activity against spleen or lymph node cells. Thus, the antisera specifically cytotoxic to plasma cell tumors and the antisera specifically cytotoxic to spleen and lymph node cells were obtained.

The antigens responsible for these specificities probably do not belong to the P_C alloantigen recently described by T. Takahashi, L.J. Old and E.A. Boyse [J. Exp. Med. 131: 1325, 1970] since the cytotoxic titers of these antisera were not reduced by absorption with liver or

kidney cells which were reported to contain P_C alloantigen.

The specificity among individual tumors was also demonstrated in the case of antiserum against MOPC-104E. The cytotoxic activity against MOPC-104E cells was hardly removed from the antiserum by absorption with the other tumors (individual or mixtures). However, the specificity was not as clear-cut with the other antisera. The cytotoxic activity of the other antisera was removed by absorption with the homologous as well as the heterologous tumors although the homologous tumor was always more effective than other tumors.

The effect of the antisera specifically cytotoxic to plasma cell tumors on antibody-forming cells in vitro and on the primary and secondary immune response in vivo has been studied. Our preliminary results indicate that the antisera are also cytotoxic to antibody-forming spleen cells, presumably normal plasma cells, as determined by Jerne's plaque assay: the formation of plaque was completely inhibited when spleen cells from mice hyperimmunized with sheep erythrocytes were incubated with the antiserum and complement prior to assay although the bulk of the spleen cells (over 90%) remain viable after this treatment.

Antibodies to Acetaminofluorene Induced Tumors.-During the period of this contract, the localization properties of radiolabeled antitumor antibodies were intensively studied with the antibodies to the acetaminofluorene induced hepatoma of rats. The ability of antibodies capable of localizing selectively in the tumor were definitely demonstrated. This was done by paired and triad radioactive iodine localization studies. Radioautography with ^{125}I -labeled antibodies and the fluorescent antibody technique were also used.

A definite difference was demonstrated between anti-hepatoma and anti-normal liver antibodies. When antibodies from both antisera are injected, they are fixed in the tumor and also the liver. Both localize on the walls of the sinusoids and the connective tissue parts of the tumor and of liver. However, it was possible to show that there are differences in the localization patterns of the two antibodies in liver and in tumor. The two types of antibodies were passed through normal rats to absorb out those components reacting with normal rat tissue. This was done by injecting the normal rats intravenously with the paired labeled mixture. After an appropriate time the rats were bled and the radioactive components still in the circulation were injected into fresh animals (normal and tumor bearing). It was found that the anti-hepatoma antibody still localized specifically in the dense connective tissue and there was no such preferential localization from anti-normal liver serum. Neither in-vivo adsorbed antibody showed any preferential localization on the walls of the sinusoid like vascular channels showing that these components in both sera, capable of being fixed in the normal liver, had been effectively removed.

In order to determine where the antigenic components reside which are important for the localization phenomenon, rabbit antibodies were prepared against the microsome and the dense sediment fractions of the hepatoma and these antibodies were compared in vivo and in vitro. Although both antibodies localized in the hepatomas and normal livers when injected into tumor-bearing or normal rats, clear differences in their localizing properties could be shown by various technics. The combined use of ^{125}I -labeled anti-microsome and ^{131}I -labeled anti-

sediment antibodies in the same mixture (paired label technic) made it possible to compare the 2 antibodies quantitatively under strictly identical conditions. The localization in vivo of the anti-microsome antibodies was greatly reduced by treatment with the hepatoma microsomes, but no effect was observed on the localization of anti-sediment antibodies. When the hepatomas from the injected rats were fractionated into subcellular fractions, it was found that the microsome fraction contained a larger proportion of the anti-microsome antibodies than of the anti-sediment antibodies, whereas the dense sediment fraction contained a larger proportion of the anti-sediment antibodies than of the anti-microsome antibodies. The 2 antibodies also behaved differently when their localization was examined as a function of time. The anti-microsome antibodies seemed to localize and be eliminated more rapidly than the anti-sediment antibodies.

These differences were clearly reflected in their sites of localization as determined by radioautography of tissue sections from rats which had been injected with ^{125}I -labeled antiserum globulin. The anti-microsome antibodies localized preferentially on the sinusoidal walls and on the hepatoma cells and normal hepatic cells. In contrast, the anti-sediment antibodies localized heavily in the connective tissues and lightly on the sinusoidal walls, but not on the hepatoma or normal hepatic cells.

Staining tissue sections in vitro by the fluorescent antibody technic showed that the anti-microsome antibodies were directed against the components of the hepatic cells and the anti-sediment antibodies against the components of the connective tissues.

Rabbit antibodies against the microsome fraction of the N-2-fluorenylacetamide-induced rat hepatoma were shown to localize in the tumorous livers (hepatoma and the remaining portion) and in normal livers when injected i.v. This was demonstrated by the radioiodine paired label technic. The localizing antibodies were purified by specific adsorption and elution. The purified product showed increased localization in the tumor livers and normal livers. It contained some components, presumably soluble complexes of hepatoma antigens and antibodies, that were separated from the 7S antibody by gel filtration. The 7S antibody, free of complexes, showed good localization. The complexes also showed some localization, but were present in too low a proportion to form an important contribution toward the total localization of the product before the gel filtration.

Antibodies against the microsome fractions of the hepatoma and the normal rat liver were further fractionated specifically on microsomes of both tissues and separated in fractions containing hepatoma-favoring or normal-liver-favoring antibodies. The results indicate differences in the antigen composition between the microsome fractions of the two tissues as well as differences in the antibody composition between the antisera produced against them.

The antiserum globulin used here was isolated by chromatography on DEAE-cellulose and was entirely 7S γ -globulin. It was freed of anti-rat plasma antibodies by a solid adsorbent containing rat plasma proteins.

Cross Reactions of Antigens in Hepatomas.-In a study with fluorescent antibody technic, 2 antisera prepared against individual nodules of 2-acetylaminofluorene-induced rat hepatoma were shown to react with the hepatoma used for immunization after exhaustive absorption with normal liver. A number of antisera prepared against individual hepatoma nodules were used. Even after exhaustive absorption with normal liver, each of the antisera reacted to some degree with all of the hepatoma nodules tested indicating that some antigens which are lacking in normal liver, or present only in undetected amounts, are present in common in all the hepatoma nodules.

A comparison of the intensity of the reaction between various antisera and hepatoma nodules, including those used for immunization, indicated that several antigenic components are present in common in the tumors, but in different concentrations in each of the tumors. These antigens were also detected in hepatoma cells grown in continuous cell culture.

In addition, the presence of some normal liver antigens in hepatoma cells was also shown by the fact that specific antisera against normal liver were able to react with hepatoma.

Immunoglobulin (IgG) Associated with Microsomes from Autochthonous Hepatomas and Normal Livers of Rats.-During the course of our investigation of the properties of rabbit antibodies produced against the microsomes of the 2-acetylaminofluorene induced tumors in rats, it was found that the antisera contained significant amounts of antibodies which reacted with IgG globulin from rat serum. On the other hand, antiserum against

normal liver microsomes, showed very little such antibody. We have now isolated the protein responsible for this reaction from the membrane portion of microsomes from autochthonous acetaminofluorene induced rat hepatomas. It is eluted with pH 2.4 glycine buffer but not by 0.25 M sucrose. Suitable purification yielded a fraction with the properties of serum IgG containing essentially no tissue protein. A similar protein was also isolated from the corresponding microsome fraction of normal rat liver but in reduced amounts.

The protein so isolated differed from the plasma IgG in its absorption properties with respect to the microsomes with tumor and normal liver. The protein appears to have been absorbed selectively from the circulation in the hepatoma. The eluted preparations that are obtained from tumor or liver were found to bind to a much greater degree in vitro to the microsomes of these tissues than was IgG isolated from serum.

The process of carcinogenesis by acetaminofluorene has been investigated by immunochemical methods and it was demonstrated that the carcinogen binds to certain components of normal liver and will fix intravenously injected anti-carcinogen antibody.

Rabbit antibody against the 2-azofluorenyl group was prepared and purified specifically by the use of a hapten-specific solid adsorbent. These antibodies, when radiolabeled and injected intravenously, were shown to localize specifically in the liver and kidney of rats previously injected with 2-aminofluorene or 2-acetylaminofluorene and also in the liver and kidney of rats fed 2-acetylaminofluorene. The antibodies did not localize in the organs of untreated normal rats or of untreated rats with tumors induced by feeding 2-acetylaminofluorene. The latter

rats had not received any carcinogen 0.5-1 year prior to the antibody injection. However, when such tumor-bearing rats were injected with 2-amino- or 2-acetylaminofluorene prior to antibody injection, the antibodies did localize in the apparently healthy portions of liver and kidney, but not in the tumor itself. When rats were injected with 2,7-diacetylaminofluorene, 2-amino-7-acetylaminofluorene, 2,7-diaminofluorene, 2,7-dinitrofluorene, and fluorene, they did not show any localization of the radiolabeled anti-2-azofluorene antibodies above control globulin.

The in vitro fixation of the anti-2-AzF antibody with frozen sections of liver from rats fed or injected with fluorenyl derivatives was quantitatively measured by the radioiodine labeled antibody technique. The anti-2-AzF antibody showed a higher fixation at earlier stages of 2-AAF feeding than at later stages and showed no definite fixation shortly after completing the 16 weeks feeding schedule. When rats on normal diet after completing the 2-AAF feeding were injected with 2-AAF and assayed, the antibody reacted more weakly with hepatomatous nodules than with preneoplastic or normal liver, indicating that the carcinogen-binding components are still present in preneoplastic stage and their deletion is prominent in hepatomatous nodules.

Cellular distribution of the carcinogen in the livers of these rats determined by fluorescent antibody technique clearly demonstrated that the carcinogen-binding components of hepatic cells are present at a particularly high concentration in the cell boundary and the perinuclear zone and that these components are partially deleted in the cells of hyperplastic nodules, which appear at the later stage of the 2-AAF feeding, and almost completely deleted in hepatomatous cells.

The anti-2-azofluorenyl antibody was shown by radioimmuno-electrophoresis experiments to be directed against the 2-azofluorenyl group since the combination of antibody with antigen was inhibited by the presence of 2-aminofluorene and to a lesser degree by 2-acetylaminofluorene and 2-amino-7-acetylaminofluorene.

Our results indicate that some components of the hepatic cell surface are able to combine with fed or injected 2-aminofluorene and 2-acetylaminofluorene and then to fix the antibody in vivo, when the antibody is injected i.v. These components are not present in the tumor.

An antigenic analysis was made of the microsome fractions prepared from livers of normal rats and rats treated with 2-aminofluorene or 2-acetylaminofluorene as well as from hepatomas induced by feeding 2-acetylaminofluorene. The microsomes were solubilized with deoxycholate and examined by immunoelectrophoresis using rabbit anti-serum against normal liver microsomes which had been absorbed with rat plasma and kidney microsomes.

At least nine precipitate arcs were detected with preparations from livers of normal rats. Similar patterns were obtained with preparations from livers of rats which had been injected with 2-aminofluorene or 2-acetylaminofluorene 72 hours earlier. In the latter case, at least 2 to 3 arcs were shown to fix ¹²⁵I-anti-2-azofluorene antibody, indicating that some microsomal components combined with the injected carcinogen.

Some of the liver microsomal antigens including one of the carcinogen binding components were not detected in preparations from livers of rats which had been fed 2-acetylaminofluorene for 16 weeks and removed from the carcinogen diet for 1, 2 and 4 weeks,

respectively.

Similar but more extensive changes in antigen composition were observed with preparations from hepatomas. Some antigens including carcinogen-binding components were not observed. In contrast, some antigens not clearly detectable in normal liver preparations were observed in hepatoma preparations."

Localization of Antibodies to Cultured Cells.-A study was made of the in vivo localization of antibodies to the lymphocytic leukemia L1210 of mice using leukemia cells grown in tissue culture for the production of antibodies. Purified rabbit radiolabelled antibodies against tissue cultures of the lymphocytic leukemia of mouse (L1210) were found to localize in vivo in L1210 solid tumors of mice, liver, kidney, spleen and lung of normal and tumor-bearing mice. The purification procedure resulted in the isolation of amounts of antibodies capable of localizing in solid L1210 tumors, which were five times the amount obtained from normal globulin by the same procedure. About 30 times as much activity was obtained from the antiserum than from normal globulin with respect to localization in spleen and liver. By suitable absorption it was found that the antibodies against the in vitro grown L1210 cells reacted with antigens in liver and spleen which are missing from kidney and lung.

Localization of Normal Serum Globulin Components.-We have also been studying components of normal globulin which show specific binding and localization properties. A part of the IgG from normal rabbit serum has been isolated which shows specificity of reaction when compared with the whole of rabbit serum IgG. When it is injected intravenously into mice bearing the L1210 leukemia it has been found to be fixed in

spleens to a much higher degree than is whole IgG. It is also fixed selectively in vitro on the sediments of L1210 cells.

Our results actually indicate the presence of several types of components in normal rabbit IgG. At least two of them show affinity for mouse tissues. One exhibits some affinity for L1210 leukemic cells, but not for kidney or spleen, while the other cross reacts with mouse kidney and spleen in vitro. The first type of component, when injected into mice, also seems to show specific localization in the spleens of leukemic mice although there is no localization in the spleens of normal mice. The presence of similar components, frequently referred to as normal or natural anti-tissue antibodies, has been repeatedly demonstrated in many sera of unimmunized normal animals. Our results show that such components may exhibit specific reactivity in vivo as demonstrated by selective fixation in particular tissues. It is not known whether the binding takes place through the part of the IgG molecules on which the antigen binding sites reside (Fab) or on the part responsible for binding to tissue in general (Fc).

It is conceivable that the selectivity of localization is due to the presence of antibody directed against an unknown immunogen. Numerous components are wide spread in the rabbit's environment and might stimulate the formation of antibodies which cross react with certain mouse tissues.

Distribution of Histocompatibility Antigens in Tissues.-This is an important problem in organ transplantation. Although the amount of an isoantigen in the tissue may play an important role, an even more important question is how accessible are the isoantigens to corresponding isoantibodies in the circulation. Previous approaches in other laboratories attempted to quantitate the amount of antigen by measurements in vitro of the capability of cells, tissue homogenates or subcellular fractions

to absorb cytotoxic or hemagglutinating isoantibodies, or measurements in vitro of the differential sensitivity of target cells to the cytotoxic action of isoantibodies. The capacity of a tissue to promote tumor homograft survival in-vivo (enhancing effect) was also used.

In our work we investigated, in the case of mice, the accessibility of the isoantigens to isoantibodies in the circulation by determining the distribution of labeled isoantibodies which are injected intravenously into mice. The isoantibodies were prepared by injecting C57BL/6 mice with ascites L1210 leukemia cells derived from DBA/2 mice. The fixation of isoantibodies in the tissues was measured by the use of the paired label radioantibody technique.

It was found that several organs showed appreciably greater fixation of the isoantibodies than of normal globulin in those mice which shared isoantigens with the strain against which the isoantibodies had been prepared.

Lung, spleen and liver fixed large proportions of the injected antibodies while kidney, thymus, lymph nodes, and heart did not fix large amounts of isoantibodies. Only minute amounts of isoantibodies were fixed in the brain, but the localization was highly specific. There was essentially no localization of normal globulin in the brain.

No localization could be detected in organs of C57BL/6 mice which served as isoantibody donors. Some cross-localization was observed in the tissues of other inbred strains, depending on their genetic relation to the DBA/2 mice.

The degree of in-vivo localization of isoantibodies does not represent the amount of the corresponding isoantigens in a certain organ, but depends mostly on the availability of these antigens to localizing antibodies.

Radioimmunoassay of Specific Antigens in Tissue Sections.-A micro-scale modification of the paired label radioantibody technique has been developed to determine the reactivity of antiserum globulin with tissue sections or cell smears. A mixture of ^{125}I -labeled IgG from antiserum and ^{131}I -labeled IgG from normal serum is applied on test specimens fixed on microscope coverslips. After incubation for 60 min at room temperature, the specimens are washed and assayed for the radioactivity due to ^{125}I and ^{131}I . The degree of the reactivity of antiserum IgG was expressed by the uptake quotient, the quotient of the specific uptake over the nonspecific uptake. ✓

The method is sensitive and reproducible under the conditions given. It is suitable for comparing a large number of specimens and particularly useful where the test materials, tissues or cells, are available only in limited quantities.

Various factors involved in the reaction--reaction time, concentration of antibody globulin, area and thickness of sections, antigen content and absorption with tissue sediments--were evaluated in detail.

This method was used to study the antibodies formed in rabbits against two cultured human leukemia cell lines, #6410 and A-LK10. Five groups of antibodies differing in their specificities were characterized.

These are: 1) antibodies specific to individual leukemia cell lines; 2) antibodies reacting with other leukemia cell lines as well; 3) antibodies reacting as in 2 and in addition with lymphoid organs and with leukocytes; 4) antibodies reacting as in 3 and in addition with liver and kidney, and 5) antibodies reacting as in 4 and in addition with nonleukemia cell lines of human origin.

Localization Properties of Anti-Moloney Lymphoma Antibodies.-Radio-

iodine labeled globulin isolated from isogeneic mice immunized with irradiated Moloney lymphoma cells, and purified by absorption on the elution from the insoluble sediment of a homogenate of the Moloney lymphoma were shown to localize in spleen of Moloney lymphoma-bearing mice. Three different Moloney lymphomas were tested. In all three of them, there was a significant increase in specific localization of the antiserum globulin in spleen of the lymphoma-bearing mice, as compared to normal mice. There was little localization in the tumors however.

Specific antibody localization was obtained in the spleen of tumor-bearing animals, which is essentially the only organ in which such localization could be demonstrated, is in accord with the tumor cell content of the spleen and the better circulation of blood through the spleen.

There are numerous possible reasons for this failure to localize in tumors. The most probable are the following: the vascular circulation through the tumor is poor in the case of the solid tumors, any specific localization hidden by the high nonspecific uptake of macromolecules by tumors (Duran-Renals, Am. J. Cancer 35: 98 (1939)), or that the pertinent antigens may not have been available to circulating antibody.

Whether the antibodies being fixed are those directed towards antigens expressed on the outer membrane of virus carrying cells or towards virion antigens remains to be seen. The latter could be present in the cell interior, on budding virus particles and as free virus. It should be borne in mind that the insoluble sediment of tumor homogenates was used as the adsorbent for the purification of the labeled antibodies in the present work and the antibodies must therefore reflect the antigenic

composition of this sediment. This may correspond more to intracellular, than to surface expressed materials. Due to the preferential multiplication of the virus particularly in the megakaryocytes of the spleen and the bone marrow (Rauscher and Allen, J. Natl. Cancer Inst. 32: 269 (1964); Moloney, Ann. Rev. Med. 15: 383 (1964)), it is conceivable that more antigen of this type is available in the virally infected spleen, than in the growing tumor where much of the corresponding antigen would be hidden behind intact cell membranes. Lymphoma cells show a variable degree of virus production by budding and the virus release in transplanted Moloney lymphomas can be quite low (Klein, Klein and Haughton, J. Natl. Cancer Inst. 36: 607 (1966)). Absorption to and elution from intact tumor cell surfaces will be required to clarify this question.

Immunosuppressive Activity of Rabbit Antisera Directed Against Mouse Lymphocytic Leukemia L1210. - In the course of studies on the in vivo localizing properties of antibodies directed against the lymphocytic leukemia L1210, it was found that the antibodies exhibited marked localization in spleens of the injected mice as did antibodies directed against homogenates of mouse spleen and lymph nodes. Such localization in spleen was seen with anti-L1210 sera prepared against cells grown in continuous culture as well as those in the ascites form.

In view of the fact that antibodies against lymphocytic cells are known to suppress antibody formation (Russell and Monaco, Transplantation 5: 1086 (1967)), it was of interest to determine whether the antibodies to cultured leukemic cells and to ascites cells would cause immunosuppression in mice.

The effect on the immune response was evaluated by determining the concentration of plaque forming cells in the spleen and by determining the titer of humoral hemagglutinins 4 days following a single injection of

sheep erythrocytes into mice.

The extent of immunosuppression was comparable to the one induced by injection of antisera directed against mouse spleen and lymph node antigens. The degree of immunosuppression was quantitated by assaying the number of antibody forming cells by plaque formation in a sheep erythrocyte medium, and by titration of humoral hemagglutinins.

The Localization of Mouse Alloantibodies of Known Specificities.-

Another study dealt with the localization of mouse alloantibodies of known specificities.

Previous studies had demonstrated specific localization in vivo of C57BL/6 anti-DBA/2 alloantibodies in DBA/2 mice (Witz, Yagi and Pressman, J. Immun. 101: 217 (1968)). In this strain combination, antibodies were presumably formed against several antigenic components of the H-2 complex and also against alloantigens of other histocompatibility (H) systems. We have now examined antisera from other mouse strain combinations for the purpose of studying separately localization of antibodies induced by H-2 and by non-H-2 transplantation antigens. Some of the antisera were directed primarily against single alloantigens of the H-2 complex or to H-1 alloantigens. We measured antibody localization in mice of strains whose tissues either did or did not contain the pertinent alloantigen.

Our results showed that (a) histocompatibility antigens of mouse tissues are exposed in vivo to humoral transplantation antibodies, and (b) antigenic tissues can readily fix such antibodies from the extracellular fluids. Antigens of the major murine H-2 system and other H systems opposing weaker barriers to allografts are responsible for the formation and organ localization of these antibodies. Localization of radiolabeled antibodies varied in amount from one organ to another,

presumably in relation with different concentrations of H antigens on the surface of various cell types (Davis and Silverman, Transplantation 6: 535 (1968); Boyse, Old and Stockert, Proc. Nat. Acad. Sci. 60: 886 (1968) and Cresswell and Sanderson, Transplantation 6: 996 (1968)), and the circulation through the organ.

Localization of transplantation antibodies was detectable even when specific binding was restricted to a single antigen of the H-2 complex. This was shown either by injecting polyvalent antisera into mice possessing only one of the possible target antigens (e.g., H-2.S), or by using monospecific sera directed against one of the antigens H-2.4, H-2.28 and H-2.31. However, antibodies directed against H-2.3, H-2.13 and H-2.27, and globulins of mice immunized against H-2 antigens did not localize in organs of appropriate mice. While it is questionable whether humoral antibodies were formed during immunization against H-1 alloantigens (Snell and Stimpfling, in Biology of the Laboratory Mouse, p. 457, (1966); Graff and Snell, Transplantation 6: 598 (1968)), the monospecific antisera did contain hemagglutinins and cytotoxic antibodies. It is possible that the antisera whose globulins failed to localize in vivo did not contain antibodies with the necessary binding properties. Alternatively, titers of localizing antibodies in these sera were so low that they were not detected above background. Another possibility is differential availability of alloantigens to humoral antibodies reflecting concentration, spacial distribution, and relationships of the antigens at the cell surface (Boyse, Old and Stockert, Proc. Nat. Acad. Sci. 60: 886 (1968); Cresswell and Sanderson, Transplantation 6: 996 (1968)).

The fact that alloantibodies localize specifically in vivo offers the means of identifying cells by antigenic markers in recipients of allografts. We have used the technique to detect early chimerism for hemopoietic allografts (Witz, Cudkowicz and Pressman, unpublished results) and others have used a similar approach to detect antibody localization in skin allografts (Perper and Najarian, Transplantation 6: 833 (1968)). Conceivably, the paired labeled method could also offer the means of identifying in vitro alloantigens of cells for histocompatibility typing. A possible advantage of this method over the more conventional serologic reactions is that firm binding of antibody to antigen is the only requirement for detection, and that the binding can occur on the surface of any cell type. Other immune assays depending on biologic effects such as changes of membrane permeability, cytolysis and agglutination require particular types of cells and thus are not of much general applicability.

Computation of Results of Paired and Triad Label Techniques.

Since the experiments in this area depend very largely on the use of radioactive iodines and since the paired label and the triad labels are essential in certain experiments, programs have been developed for the computation of results using ^{131}I , ^{125}I and ^{130}I as a triad and two of the iodines as a diad.

EVALUATION OF PROGRESS

The original objective of these investigations was to obtain antibodies capable of being fixed in spontaneous and induced tumors. We have been able to show that such antibodies are formed. We have been able to analyze where they localize and we have been able to fractionate tumor tissues and also to separate the antibodies into fractions showing selective reactivity with particular components. We have shown that the antigens responsible for fixing the antibodies are present in several parts of the tumor and that the most specific antibodies are directed against the microsomes which are part of the cell surface.

Specific localization does take place. Methods to increase the localization have to be developed. We have shown that there are individual differences between the individually induced tumors. Individual differences were also found in plasma cell tumors of mice.

We have also demonstrated possible mechanisms for the carcinogenic activity of the carcinogen, acetaminofluorene and shown the effect of this carcinogen in deleting components of the liver. In addition to the demonstration of localization in tumors we have been able to show that other antigens of importance can fix the specific antibodies in vivo when injected. For example Moloney lymphoma cells in the spleen fix antilymphoma antibodies and various tissues fix isoantibodies in mice.

BIBLIOGRAPHY
(Papers supported by this contract)

Publications associated with this contract are the following:

1. Pressman, D. Labeled Antibodies in Autochthonous Tumor Studies. *Cancro*, XIV, (1961).
2. Pressman, D. and Kyogoku, M. The Localization In Vivo of Rabbit Antibodies in the N-2-FAA Hepatic Tumors of Rats. *Ann. N.Y. Acad. Sci.*, 101: Art. 1, 167 (1962).
3. Kyogoku, M., Yagi, Y., Planinsek, J., Bernecky, J. and Pressman, D. Localizing Properties of Anti-Rat Hepatoma Antibodies In Vivo. *Cancer Res.*, 24: 268 (1964).
4. Isojima, S., Bernecky, J., Planinsek, J., Yagi, Y. and Pressman, D. Differences Between Antibodies against the Dense Sediment and Microsome Fractions of the N-2-Fluorenylacetamide-Induced Rat Hepatoma. *Cancer Res.*, 25: 968 (1965).
5. Isojima, S., Yagi, Y., Planinsek, J. and Pressman, D. Purification of Localizing Antibodies Formed against Microsomes of the N-2-Fluorenylacetamide-Induced Rat Hepatoma. *Cancer Res.*, 25: 962 (1965).
6. Pressman, D. and Pressman, R. Computer Programs for Paired and Triad Radioiodine Label Techniques in Radioimmunochemistry. *Intl. J. Appl. Rad. Isotopes*, 16: 617 (1965).
7. Pressman, D. and Pressman, R. Computer Programmes for Multiple Labelling Techniques Involving Two or More Radioiodines in Radioisotope Sample Measurement Techniques in Medicine and Biology, 223 (1965).
8. Kitagawa, M., Yagi, Y., Planinsek, J. and Pressman, D. In Vivo Localization of Anticarcinogen Antibody in Organs of Carcinogen-treated Rats. *Cancer Res.*, 26: 221 (1966).

9. Kitagawa, M., Tanigaki, N., Yagi, Y., Planinsek, J. and Pressman, D. Carcinogen-binding Antigens in Rat Liver Microsomes. *Cancer Res.*, 26: 752 (1966).
10. Isojima, S., Planinsek, J., Yagi, Y. and Pressman, D. Differences Between the Microsomes of Normal Rat Liver and of N-2-Fluorenyl-acetamide-induced Rat Hepatoma as Determined by the Paired Label Antibody Technic. *Cancer Res.*, 26: 1527 (1966).
11. Tanigaki, N., Yagi, Y. and Pressman, D. Application of the Paired Label Radioantibody Technique to Tissue Sections and Cell Smears. *J. Immunol.*, 98: 274 (1967).
12. Tanigaki, N., Yagi, Y., Moore, G.E. and Pressman, D. Antibodies Against Human Leukemia Cell Lines. *J. Immunol.*, 98: 281 (1967).
13. Tanigaki, N., Kitagawa, M., Yagi, Y. and Pressman, D. The Reaction of Specific Antibody with 2-Acetylaminofluorene Fixed in Liver Cells. *Cancer Res.*, 27: 747 (1967).
14. Yagi, Y. Use of Radioiodine in Immunological Studies of Tumors in Radioisotopes in Medicine--In Vitro Studies, Edited by R.L. Hayes, F.A. Goswitz and B.E. P. Murphy, Springfield, Virginia, AEC 13 Symposium Series, 607-619, 1968.
15. Witz, I., Yagi, Y. and Pressman, D. IgG Associated with Microsomes from Autochthonous Hepatomas and Normal Liver of Rats. *Cancer Res.*, 27: 2295 (1967).
16. Witz, I., Yagi, Y. and Pressman, D. Immunosuppressive Activity of Rabbit Antisera Directed Against Mouse Lymphocytic Leukemia L1210. *P.S.E.B.M.*, 127: 562 (1968).
17. Witz, I., Yagi, Y. and Pressman, D. Availability of Isoantigens to Circulating Antibodies. *J. Immunol.*, 101: 217 (1968).

18. Witz, I., Yagi, Y. and Pressman, D. A Normal Component in Rabbit IgG with Affinity for Mouse Tissues. *Immunology*, 15: 765 (1968).
19. Witz, I., Kaliss, N. and Pressman, D. Isoantigens Available to Circulating Antibodies. *J. Immunol.*, 102: 283 (1969).
20. Isojima, S., Yagi, Y. and Pressman, D. Antigens Common to Rat Hepatomas Induced with 2-Acetylaminofluorene. *Cancer Res.*, 29: 140 (1969).
21. Witz, I., Yagi, Y. and Pressman, D. Brain Localizing Activity of Hetero- and Isoimmune Sera Directed Against Lymphocytic Cells. *P.S.E.B.M.*, 130: 928 (1969).
22. Witz, I., Klein, G. and Pressman, D. Moloney Lymphoma Antibodies from Mice; Localization in Spleens of Moloney Lymphoma Bearing Mice. *P.S.E.B.M.*, 130: 1102 (1969).
23. Witz, I., Cudkowicz, G. and Pressman, D. Availability of Transplantation Alloantigens to Circulating Antibodies. Studies with Alloantibodies of Known Specificity. *J. Immunol.*, 102: 1373 (1969).
24. Witz, Isaac, Klein, George and Pressman, David. Moloney Lymphoma Antibodies from Mice; Localization in Spleens of Moloney Lymphoma Bearing Mice. *Proceedings of the Society for Experimental Biology and Medicine* 130: 1102, 1969.
25. Witz, Isaac, Cudkowicz, Gustavo and Pressman, David. Availability of Transplantation Alloantigens to Circulating Antibodies: Studies with Alloantibodies of Known Specificity. *Journal of Immunology* 102: 1373, 1969.
26. Witz, Isaac, Cudkowicz, Gustavo and Pressman, David. Detection of Hemopoietic Allografts by Localization of Radiolabeled Alloantibodies. *Journal of Immunology* 104: 19, 1970.

27. Watanabe, Takeshi, Yagi, Yasuo and Pressman, David. Antibody Against Neoplastic Plasma Cells. I. Specific Surface Antigens on Mouse Myeloma Cells. The Journal of Immunology 105: 1213 May 1971.
28. Watanabe, Takeshi, Yagi, Yasuo and Pressman, David. Antibody Against Neoplastic Plasma Cells. II. Suppressive Effect on Antibody-Producing Cells. Journal of Immunology 107: 1706 (1971).
29. Pressman, David. Isolation and Properties of HL-A Antigens and Antigens of Neoplastic Mouse Plasma Cells. In CELLULAR ANTIGENS (ed. Alois Nowotny), Springer-Verlag/New York, 1972.
30. Kitano, M., Mihich, E. and Pressman, D. Antigenic Differences between Leukemia L1210 and a Subline Resistant to Methylglyoxal-bis (guanyldiazotone). Cancer Research 32: 181 (1972).

STATE OF KNOWLEDGE IN THIS AREA

We now know that antibodies can be prepared which localize in certain tumors. There is some cross reaction with normal tissue but there appear to be definite differences in antibody to tumor and to normal tissue. Use of radiolabeled anti-tissue antibodies has shown very definitely that the vascular beds in various normal organs and in tumors are different. There are components in common in the vascular beds. However the vascular beds do have individual differences depending upon the particular organs through which they are passing. These differences represent different components in contact with the circulation. Antibodies directed against these individual components show preferential localization in the individual tissue. These have been shown for tumors and for several normal tissues.

The radiolabeled in vivo localization method is a powerful tool for the analysis of the chemical structure of the features involved.

These chemical structures need to be evolved since they may give leads as to the basic defects occurring in various vascular diseases.

They also may be important factors in the immune response to transplantation of tissues and organs.

The application of radioantibodies to tumor is very pertinent to the use of any serum therapy of tumors since the fixation of antibodies can be measured quantitatively and a determination made of whether the antibody reaches the tumor or not.