11. Progress Report on Work in Past Year

CALI-7676-x4

A three-year comprehensive summary is submitted along with this application. Following are somewhat more detailed summaries of work conducted specifically during the past year.

THE MITOGENIC ACTIVITY OF A C57BL/10 MOUSE
TRANSPLANTABLE LYMPHOMA

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Investigators: Bob G. Sanders, Ray L. Teplitz*, Margaret Wilkie

A C57BL/10 transplantable lymphoma has been carried in our laboratory for the past four years through transfer of spleen cells to inbred C57BL/10 mice. Histological studies revealed infiltration of the hemopoietic organs of the lymphomatous mice with reticulum cells. Subsequently, it was observed that a mitogenic factor is associated with the lymphoma. The purpose of this study was threefold: to investigate the nature of the mitogenic factor, to correlate histological findings with the mitogenic activity, and to determine the growth and mitogenic action of the lymphoma in mice of different histocompatibility types.

The lymphoma was found to stimulate waves of partially synchronized host-cell multiplication in the spleens of lymphoma-injected mice (Sanders, 1966). This mitogenic action of the lymphoma was not limited to living cells, but was found also in X-ray inactivated cells, and in the supernatant from centrifuged suspensions of these cells. The mitogenic factor was not erythropoietin. It did not appear to reflect an antigenic stimulation of the host-spleen response.

The histological studies correlate well with the isotope studies. The initial peak of DNA synthesis (after transfer of the mitogenic factor to the host) at 72 hours may be correlated with the rapid expansion of the germinal centers of the host, reflecting considerable proliferative activity. This peak is followed by a drop in DNA synthesis (day 5), which is seen histologically as a plateau of activity. DNA synthesis peaks again at day 7, presaging the rupture of the follicular boundary by the tumor which is completed by the twelfth or fourteenth day.

The lymphoma transgressed H-2 histocompatibility barriers and it killed hosts of the line of origin more rapidly than it killed hybrid mice one of whose parents is the line of tumor origin.

* City of Hope Medical Center, Duarte, California.

References:

Sanders, Bob G. (1966) Biology Annual Report, No. 37.

Sanders, Bob G., Teplitz, Ray, and Wilkie, Margaret (1968) (Submitted for publication to Cancer Research).

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INVESTIGATION OF THE "NATURALLY" OCCURRING ANTIBODIES

Investigators: Bob G. Sanders, William Murphy, David Muchmore *

The "naturally" occurring human anti-B isoantibodies have been reported (Wurmser et al., 1954) to be different according to the individual in which they are produced. For example, the molecular weight has been estimated to be 500,000 for $\beta(A_1O)$, 300,000 for $\beta(A_1A_1)$ and 170,000 for $\beta(OO)$. It has been reported (Owen, 1954) that sera from individuals of "O" blood type contain an antibody that cross-reacts with A and B red blood cells. The purpose of this study was to check and extend the studies of Wurmser and coworkers, and to investigate the nature of the cross-reacting antibody.

Sera were obtained from individuals of $\beta(A_1O)$ and $\beta(OO)$ blood types. The sera, purified IgM, and IgG were assayed by agglutination and hemolysis for biological activity against B red blood cells. The two sera differed in their hemolytic and agglutinating ability. The β antibodies from OO serum agglutinated and hemolyzed B cells; the β antibodies from A_1O serum produced agglutination only. Upon fractionation of the serum by gel filtration, peak 1 (IgM) and peak 2 (IgG) from OO serum gave agglutination and hemolysis. Only peak 1 (IgM) from the A_1O individual agglutinated the B test cells. The data, although not conclusive, suggest that the naturally occurring β antibodies from A_1O serum are of one class, whereas OO serum has two classes of such antibodies. Our studies differ from Wurmser's in that the OO serum contains naturally occurring antibodies of both the IgM and IgG varieties, as determined by gel filtration.

The IgG antibodies from OO serum were further purified by DEAE Sephadex column fraction, and checked for purity by immunoelectrophoresis. The purified IgG, as well as IgG remaining after absorption with A, B, O, and AB red blood cells were tested by hemolysis and agglutination for biological activity. The unabsorbed IgG agglutinated and hemolyzed (with complement from an AB person) A, B, and AB red blood cells. The IgG remaining after absorption with AB cells did not react with the test cells, whereas the IgG absorbed with O red blood cells reacted with test cells as expected. Absorption of the IgG with B red blood cells left antibodies that reacted with A and AB test cells; however, absorption with A cells removed the antibodies to A, AB, and B test cells. ✓

The studies demonstrate that the OO serum contains natural blood group antibodies of IgM and IgG varieties. The A_1O serum contains only one variety, probably IgM. With absorptions, it is possible to fractionate the IgG "naturally" occurring antibodies of OO serum into two groups - one reacting specifically with A test cells, and the other cross-reacting with A and B test cells.

The role of the "naturally" occurring IgG antibody in ABO incompatibility is of great interest. It is known that the IgG globulin can pass the placenta barrier. It is conceivable that the "naturally" occurring IgG antibodies (specific for A cells as well as the A-B cross-reactive antibody) cross the placenta, and if so, they could be directly involved with the ABO incompatibility.

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References:

- Wurmser, S. F. et al. (1954) *Annals of Eugenics* 18: 183.
- Owen, Ray D. (1954) *J. Immunology* 73: 29.

AN ANTIGENIC COMPONENT COMMON TO KAPPA, LAMBDA
AND GAMMA POLYPEPTIDE CHAINS

Investigators: Bob G. Sanders, Leroy E. Hood, Margaret Wilkie

On the basis of homology among the amino acid sequences of myeloma light chains for mice and humans, it has been postulated that the polypeptide chains are evolutionarily related, with a polypeptide chain of about 100 amino acids being the fundamental unit of all chains (Lennox and Cohn, 1967). Four nonidentical but related units connect linearly to make up a heavy chain, and two units a light chain.

The purpose of this study was to produce antisera against human myeloma light chains (κ and λ) in animals where one light chain class was absent, with the expectation that the antibodies produced in such animals would recognize gross differences as well as the subtle differences recognized by animals containing both light chain classes. Such antisera, hopefully, would recognize the presence of κ and/or λ in immunoglobulin from nonrelated species of animals.

Only κ has been detected in chicken sera; only λ has been found in horses. Antisera against human λ myeloma light chains (HBJ2) were produced in chickens, and an antiserum against human κ myeloma light chains (HBJ3) was produced in a horse. To determine reactivity, immunodiffusion tests were performed. The chicken antisera to HBJ2 reacted with human λ light chain preparations as well as with normal IgG and the light chains from many other vertebrates. These antisera to HBJ2 further recognize a common antigenic component shared between κ and λ light chain preparations. Inhibition with κ light chains rendered the antisera reactive only to λ , but with a reduced titer. Further, inhibition with heavy chains from completely reduced and alkylated IgG rendered the HBJ2 antisera nonreactive to κ light chains. The horse antiserum produced against HBJ3 reacted only with human κ myeloma light chains and with human IgG and its derived light chains. The studies with the antisera to λ light chains, produced in chickens, suggest that the antisera recognize a common antigenic component shared among human κ , λ , and γ polypeptide chains, as well as light chains from various other species of animals.

References:

- Lennox, E. S., and Cohn, M. (1967) *Ann. Rev. Biochem.* 36: 365.

COMPARISON OF CARBOHYDRATE-PEPTIDES FROM HEAVY
CHAINS OF IgG AND IgA

Investigators: Bob G. Sanders, Margaret Wilkie

Carbohydrate (CHO) has been found in all immunoglobulins, but the amount varies and is associated with the class of immunoglobulin. IgG contains approximately 2-3% CHO; IgA and IgM contain approximately 10%. In general, the CHO is associated with the heavy chains of the three globulins; however, CHO has been reported

to be associated with some light chains (Melchers et al., 1967).

Amino acid composition studies of the CHO-peptides eluted from heavy chains of 15 different animals demonstrated the CHO-peptide to be very similar, consisting of 9 amino acids; with the only amino acid difference a tyrosine-isoleucine interchange (Howell, Hood and Sanders, 1968). The CHO associated with the gamma (γ) chain is found in the region of the globulin molecule (Fc) associated with biological functions such as placental transfer, complement fixation, and skin fixation, and might therefore explain the high degree of sequence conservation. The purpose of this study was to isolate the CHO-peptide from the heavy chains of IgM (μ) and IgA (α), to determine the amino acid composition, and compare the findings to those of IgG (γ) in an attempt to establish an evolutionary relationship among the three heavy chains.

IgM and IgA (myeloma) were purified from human sera using a combination of DEAE cellulose ion exchange, ammonium sulfate precipitation, low ionic salt precipitation, density gradient ultracentrifugation, starch block electrophoresis, and gel filtration. The proteins were tested for purity by immunoelectrophoresis. The purified proteins were chain-separated in guanidine hydrochloride, aminoethylated, enzymatically digested, chromatographed, electrophoresed, and stained with ninhydrin to locate the CHO-peptide. One set of maps was stained lightly, the peptides were cut out, eluted, hydrolyzed, and analyzed by high voltage electrophoresis on paper.

The CHO-peptide associated with the heavy chain from IgM was not found. It is possible that the CHO-peptide is insoluble and remained at the origin. The α (IgA) heavy chain map was distinct from the γ (IgG) heavy chain. However, the CHO-peptide migrated chromatographically and electrophoretically in the identical location of the CHO-peptide from the γ chain. The CHO was found in two separate peptides, as has been reported with γ chain (Howell, Hood and Sanders, 1968). However, the amino acid compositions of the two peptides were identical, which suggested that the sialic acid difference influenced the electrophoretic mobility. IgG contamination was ruled out when the purified IgA reacted with antisera specific for IgA but failed to react with antisera specific for IgM or IgG.

The amino acid composition of the α CHO-peptide was strikingly similar to the γ CHO-peptide. However, the α CHO-peptide did not contain the amino acids phenylalanine and tyrosine but did have valine and possibly proline. They were otherwise identical.

The data suggest an evolutionary relationship between α and γ heavy chains and indicate that the region associated with the carbohydrate has changed little in evolution.

References:

- Howell, J. W., Hood, L., and Sanders, Bob G. (1967) J. Mol. Biol. 30: 555.
 Melchers, F. et al. (1966) Biochem. Biophys. Res. Comm. 24: 244.

THE EFFECTS OF ANTIGEN DOSAGE ON IgM ANTIBODY PRODUCTION

Investigators: Bob G. Sanders, Paul M. Thomas, Sandra Webb, Paul Bolen*

In previous reports (Biology 1965, 1966) it was demonstrated that IgM from rabbit antisera to T2H bacteriophage stabilizes the phage in the presence of indole, leading to the infection of the bacterial host. The purposes of this study were to determine the phage disappearance curve, and to determine the effects of antigen dosage on the onset, plateau, and decline of the antibody response.

Chickens were injected with 10^3 , 10^6 , 10^9 , or 10^{12} T2H bacteriophage in a total volume of 1.0 ml. Serum was removed at intervals of 45 minutes, 12 hours, and at each 24 hour interval for 10 days. The antisera were assayed for phage disappearance and phage activation as described previously (Biology 1965).

The disappearance of the phage from the serum of chickens was different from that expected for a primary antibody response. T2H phage could be detected only in the chicken receiving 10^{12} phage. Forty-five minutes after the injection of 10^{12} phage, only 10^6 phage were detected. Twelve hours after the injection, only 10^3 phage were detected. The phage had disappeared from the serum within 24 hours after the injection. The rapid elimination of the phage from the serum was unexpected for a primary response. It is conceivable that the chickens have a low level of antibody against T2H and the disappearance curve observed is for a secondary rather than a primary antibody response.

Sera taken at different time intervals were tested for phage-stabilizing properties with the following results:

(a) 10^3 phage dosage - IgM response was not detected, suggesting that the antigen dosage was too low to provoke a measurable response.

(b) 10^6 phage dosage - a low level of IgM was detectable on day 3. The titer increased on day 4 and peaked on day 5, at low titer. It declined on days 6 and 7.

(c) 10^9 phage dosage - a low level of IgM was detected on day 2. This rose to a high level of IgM on day 3, and peaked on day 4, one day earlier than for the 10^6 phage dosage, with an antibody titer 20 times greater than the 10^6 response. There was a slight decline on day 5, and further decline on days 6 and 7.

(d) 10^{12} phage dosage - the IgM response was essentially identical to the response provoked by 10^9 phage, suggesting that an antigenic threshold had been reached.

The phage activation studies reported were consistent with those expected for IgM, since the IgM response is known to be dependent on the antigen dosage. The studies further support the hypothesis that IgM is responsible for stabilizing the T2H bacteriophage in the presence of indole.

* NSF Summer Trainee.

References:

Marinkovich, V. A., Edgar, R. S., and Sanders, B. G. (1965) Biology Annual Report, No. 67.

Sanders, B. G., Edgar, R. S., and Marinkovich, V. A. (1966) Biology Annual Report, No. 36.

COMPARATIVE ANALYSIS OF IgG LIGHT CHAINS

Investigators: Leroy E. Hood, Bob G. Sanders

We reported in Biology 1967 a comparative peptide map analysis of heavy and light chains of IgG from 15 different animals in which a high degree of structural similarity among polypeptide chains was detected. This report is a continuation of those studies.

Peptide map comparisons were made on normal light chains from man and from other animals - rhesus monkey, baboon, sheep and cow. The chains were aminoethylated, enzymatically digested, chromatographed and electrophoresed. Preparative peptide maps were used to determine similarities and to obtain major peptides in significant amounts for qualitative amino acid analysis. The peptide maps revealed the following:

(a) Approximately 25 peptides can be detected on the human peptide map. This is approximately equal to the number of basic residues (i.e., lys, arg, and aminoethylated cys) in the common regions of λ and κ chains, and suggests that little if any of the variable region is visualized on the maps.

(b) The maps from the three primates share many common peptides. The more closely related species, monkey and baboon, have virtually identical peptide maps (one distinct peptide difference) whereas both differ from human by 5 to 10 peptides.

(c) Sheep and cow have many common peptides. Sheep share two peptides with man, and cow shares only one. The peptide maps of sheep and cow are simpler than those of primates, with fewer total peptides and less smearing and crowding of peptides having similar electrophoretic and chromatographic properties. These data suggest that sheep and cattle produce fewer classes of light chains.

Qualitative paper amino acid analysis of the eluted light chain peptides from the five species revealed the following:

A comparison of the amino acid composition of peptides from normal human pooled light chains with the human myeloma sequence of the κ and λ common regions shows that only four of twenty peptides are not from the common regions. One peptide (ala, pro, lys) could represent a variable region (region 43-45) from the most common subclass; another peptide (val, gly, lys) could not be placed in any of the light chain sequences published to date. One peptide (ala, try, lys) could be an allotypic difference if the val at the CL (147) in some of the population were present as a basic residue (e.g., CK 191 val-leu or CL 190 arg-lys interchange). The switch peptide [SK (104-107)] of human myeloma was present in the peptide maps of sheep and human pooled light chains.

The data show that peptide map analysis of light chains is limited primarily to the common region, and is of very little value in studying the variable region of heterogeneous proteins. The common regions of light chains from closely or distantly related species show identical peptides, possibly suggesting biological constraints to evolutionary changes. Peptide map studies are also useful for assaying peptide chains for allotypic or polymorphic differences.

Reference:

Hood, L., and Sanders, Bob G. (1967) Biology Annual Report, No. 186.

ROLE OF A COFACTOR IN THE ABILITY OF IgM TO STABILIZE T2H BACTERIOPHAGE

Investigators: Paul M. Thomas, Bob G. Sanders

Previous studies have demonstrated the ability of IgM to stabilize T2H bacteriophage in the presence of indole (Biology 1966). It has also been shown that this stabilization is affected by a cofactor that is labile to heating at 56°C for 30 minutes. The objective of the studies reported here was to characterize this cofactor.

It was stated (Biology 1967) that inactivation at 56°C for 30 minutes reduced the stabilizing property by approximately half. We found that in fact the degree of loss is dependent on the age of the antisera and/or repeated freezing and thawing. This loss reduced stabilization by 12% for "new" 4 day chicken antiserum, and 60% for antiserum a year old. The antisera were thawed and refrozen for each experiment, and this may have had some effect. Styk and Hana (1965), determining titers of anti-influenza antibodies in a hemagglutination-inhibition test, reported that when normal serum was added to heat-activated antiserum, titers were restored to over 100% of the original values. The test involves a cofactor, which these investigators believe not to be complement. We propose that the observed increase in activation is not true for "new" antisera, but is true when "new" normal serum is added to "old" antiserum. The normal serum adds back some aspect of the "cofactor" loss due to aging, and the resulting level is more than 100% of that of the "old" antiserum alone.

To test whether this cofactor is complement, in addition to heat inactivation which affects C'1 and C'2 extensively, the effects of EDTA, zymosan, and ammonia on the stabilization level were tested. EDTA (0.1 M) reduced the stabilizing level by 99.4%. This concentration of EDTA also completely inhibits hemolysis. EDTA is specific for C'1 in the complement system. Zymosan, which removes C'3, reduced the stabilizing level as follows: 10.0 mg/ml (38%), 1.0 mg/ml (10%), and 0.1 mg/ml (little effect). No hemolysis occurred with the first two concentrations, but slight hemolysis was evident with the third. NH_4OH , which inactivates C'4, was tested with the following results: 0.25 N (100% reduction in stabilization), 0.025 N (97%), and 0.0025 N (13%). All of these concentrations of NH_4OH prevented hemolysis.

Age of a chicken makes a difference in the effectiveness of this cofactor. Adult normal chicken serum loses very little of its stabilizing property at a 1:12,800

dilution. In comparison, although undiluted serum from a 9 day old chick is about as effective as that of an adult, at a 1:100 dilution the stabilizing effect has dropped to about background level.

While some chemical and physical analyses remain to be carried out, it is tentatively concluded that this cofactor is complement. If so, this is a very sensitive assay system for it.

Reference:

Styk, B., and Hana, L. (1965) In: J. Sterzl (ed.), Molecular and Cellular Basis of Antibody Formation, p. 261.

PRODUCTION OF IgM BY THE THORNBAC (GUITARFISH)

Investigators: Paul M. Thomas, Bob G. Sanders, Moises Eisenberg

IgM is the major and possibly the only immunoglobulin detected in the lower vertebrates. 7S globulin with a μ heavy chain has been reported for the lemon shark by Clem and Small (1967). Using an assay system in which IgM stabilizes T2H bacteriophage against the effects of indole (Biology 1965), we tested the ability of the thornback, an ovoviviparous elasmobranch, to synthesize IgM. Elasmobranchs are immunologically important in that their evolutionary position is where a rapid increase occurs in amount and complexity of lymphoid tissue (Good et al., 1966).

IgM was synthesized by these animals in response to the phage. The titer, however, was about 1/40 that of a chicken injected with the same concentration of the same antigen. Gel filtration chromatography, using a different phage (ϕ X174), indicated that the total IgM increase due to a single injection of antigen (1×10^{12} pfu) was 60-400% over background. In chickens this increase is usually less than 5%, but in some instances titers, after a single injection, have been increased 10 to 20 times in warm-blooded animals. Phage disappeared from the system in the thornback in 7-10 days. The disappearance is more rapid in chickens and mammals, and this clearing of the phage from the system is an additional suggestion of an immune response.

Immunoelectrophoresis and immunodiffusion data indicated an absence of IgG. Also, the guitarfish lack the "cofactor" present in higher vertebrates.

It is concluded that these elasmobranchs do produce IgM but in much lower titers than for higher vertebrates. The animals were kept at 19°C, a higher temperature than their natural environment. Since IgM synthesis is temperature-dependent, these animals may have the mechanism for IgM production but the temperature of their natural habitat may be too low for the production of great amounts. This may explain the low total IgM background and the great increase in total IgM when an antigen is injected into the animals maintained at higher temperatures.

References:

Clem, L. W., and Small, P. A. (1967) J. Exptl. Med. 121: 893-920.

Good, R. A., Finstad, J., Pollara, B., and Gabrielsen, A. (1966) Morphologic studies on the evolution of the lymphoid tissue among the lower vertebrates. In: R. T. Smith, P. A. Miescher, and R. A. Good (eds.), Phylogeny of Immunity. University of Florida Press, pp. 149-170..

Sanders, B. G., Edgar, R. S., and Marinkovich, V. (1965) Biology Annual Report, No. 67.