the result of congenital maldevelopment of the middle ear and not of the inner ear, as is generally supposed. In order to stimulate and improve the residual hearing in these children treatment should therefore begin at the earliest possible age with the use of a bone-conducting hearing-aid.

I am greatly indebted to Professor R. D. Lockhart for his encouragement and advice during this investigation, to Mr. I. S. D. Thomson for allowing me access to his notes on Cases 1 and 2, and to Miss J. C. Macrae for carrying out all the audiometry examinations.

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HYDATID COMPLEMENT-FIXATION TEST: INFLUENCE OF INTERMEDIATE HOST PROTEIN ON SPECIFICITY OF SHEEP HYDATID FLUID ANTIGEN

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Kellaway and Fairley (1932) considered the hydatid complement-fixation test to be specific, and we (Bensted and Atkinson, 1953), reporting on the examination with standardized reagents of a small series of cases, fully supported this opinion. Since that time a further series of over 300 cases of suspected hydatid disease have been examined serologically by the same technique, and out of 70 cases, subsequently proved to be hydatid disease, significant complement-fixing antibody was demonstrated in the serum of 61 of the patients concerned. There were 240 patients in whom further investigation showed that their condition was due to some disease other than hydatid. Sera from all but one of these patients gave negative results in the hydatid complement-fixation test. It is this false-positive result—the first in our experience—that initiated the investigations recorded in this communication. The explanation proved to be simple, and once the facts were known the results of the investigations were in full accord with what was expected. Nevertheless some interesting observations were made which seemed worthy of record as they had not, so far as is known, previously been published.

During radiological examination the patient in question had been found to have a suspicious shadow in the lung, and blood serum had been forwarded for the C.F.T. to exclude hydatid disease. The Casoni test had been reported as negative, but the C.F.T. with the standardized sheep hydatid fluid antigen was positive to a titre of 1 in 16. Further clinical investigations, however, revealed that the patient had a carcinoma of the lung. The disease ended fatally in a short time and no evidence of a hydatid cyst was found.

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It was at first thought that the complement fixation might have been due to the development of some non-specific reacting substance associated with the rapidly growing neoplasm, but our records showed that 12 similar cases of carcinoma of the lung had been examined serologically for the presence of hydatid complement-fixing antibody and in all the test had been completely negative. Further inquiries, however, revealed that the patient had recently returned to this country from the Middle East, where, a few weeks before sailing, he had been bitten by a rabid dog and had been given a "full course" of a sheep-brain anti-rabies vaccine.

Some time after this incident, and additional to the series referred to above, a similar case came to light. In this case, however, the anti-rabies vaccine had been given some years previously. Two Casoni tests gave atypical delayed reactions, and the C.F.T. performed after a short interval showed partial fixation at a titre of 1 in 2 only. The case subsequently proved to be carcinoma of the lung.

Without the history of the previous case these results would doubtlessly have been disregarded, but now the possibility of sheep-protein sensitization from the sheep-brain anti-rabies vaccine and the boosting effect of the intradermal injections of the sheep hydatid fluid (for the Casoni tests) seemed to warrant further investigation.

Although there was little doubt that individuals recently immunized with sheep-brain anti-rabies vaccine would have some sheep-protein antibodies in their sera it would be necessary to check this. There was considerable delay before we were able to obtain material for such tests, and in the meantime we decided to try to obtain the required evidence from animal experiments.

Experimental

Rabbits

Rabbits are not suitable for immunization experiments with sheep-tissue vaccines. Friedemann (1917) had shown that rabbits possess a natural haemolysin for sheep's red blood cells, and by the use of suitable antigens it was found that other sheep antibodies, such as complement-fixing antibodies for sheep protein, could be demonstrated in the sera of a high proportion of normal rabbits. The presence of this natural complement-fixing antibody in the serum of normal rabbits suggested that further examination of this point might yield information of interest.

Hiles (1926) had noted that the sera of normal rabbits gave positive complement fixation with sheep hydatid fluid and assumed that the reaction was due to some anti-hydatid substance in the sera. We confirmed Hiles's observations, but when we tested a representative number of sera against ovine and bovine hydatid fluids† and a suspension of normal sheep's brain in parallel, we found that practically all the sera showed some degree of fixation of complement with the sheep-brain antigen, but that only those sera with a relatively high anti-sheep titre gave fixation with an ovine hydatid fluid. Reactions with the bovine hydatid fluid were only occasionally seen. When complement fixation occurred with the sheep hydatid fluid the titre was significantly lower than that against the sheep-brain antigen; the occasional reactions with the bovine fluid were incomplete. Rabbit sera did not react with pure hydatid antigen, and absorption of a serum, giving fixation with both sheep-brain and sheep hydatid antigens, with massive doses of washed scolices from a fresh hydatid cyst failed to reduce the complement-fixation titre to either antigen. The titre of a serum containing true hydatid antibody was reduced to nearly zero in a parallel absorption test.

†Diluted in accordance with assay requirements.

It seemed clear, therefore, that the reactions observed by Hiles were due to the natural sheep antibody in the rabbits' sera fixing complement with the sheep protein in the ovine hydatid fluid and not to hydatid antibody. The reactions we obtained with the bovine fluid were not unexpected, since Schiff (1937) showed that there was a partial antigenic relationship between ovine and bovine tissue extracts. The explanation for the difference in the complement-fixation titres obtained with the two types of sheep antigens is discussed below.

Guinea-pigs

Natural sheep antibodies are not found in the sera of guinea-pigs, and our previous experience in the preparation of the standard hydatid antibody suggested that the animals would be suitable for immunization with a normal sheep-tissue vaccine. After pre-inoculation test-bleeds, two guinea-pigs were given weekly intraperitoneal injections of a 5% saline suspension of fresh sheep brain which had previously been well washed to remove as much blood as possible.

The antibody response was not so good as had been expected, and the final serum pool had a homologous titre of little more than 1 in 8, but the fixation was good and the end-point clear-cut. The serum, however, showed no complement fixation with sheep hydatid fluid. It would appear that the concentration of sheep protein in this fluid, as diluted for the hydatid C.F.T. by the standard method, was too low to detect the relatively low-titre sheep antibody. (We have found that when the titre of natural sheep antibody in rabbit sera had a level of 1 in 32 or more we obtained fixation with the hydatid fluid in a serum dilution of about 1 in 8.)

In order to test the possibility of multiple Casoni tests influencing antibody production in immune animals the following experiment was done. One month after bleeding the immunized guinea-pigs, when their sheep antibody level was beginning to fall, they were given spaced doses of 0.1 ml. of sheep hydatid fluid intradermally. There were no tissue reactions to the injections, but the anti-sheep titre increased by a full step to 1 in 16. This level was apparently still too low, because no reaction was obtained with the hydatid antigen. It was not feasible, unfortunately, to continue this experiment.

Human Volunteers

"Anti-rabies treatment" with the Semple type of vaccine involves a long course of daily injections of a phenolized suspension of infected sheep brain, and it seemed reasonable to expect a long-lasting "basal immunity" to sheep protein in individuals who had received this form of treatment. One of us (H.J.B.) and four colleagues, all of whom had received one or more courses of the Semple vaccine many years previously, were given intradermal injections of sheep hydatid fluid in doses of 0.1 ml. at intervals of 7-10 days to ascertain (1) if previous exposure to sheep protein influenced the response in the local reaction to the intradermal injections, and (2) whether these injections gave rise to sheep antibody response, as in the case of the guinea-pigs. The results of this experiment are summarized in Table I.

It will be noted that three of the five subjects developed significant complement-fixing antibody to sheep protein as a result of the intradermal injections of the sheep hydatid fluid.

The result of the final C.F.T. in subject P. with the hydatid antigen is of very doubtful significance. The test was twice repeated, and on each occasion there was merely delayed haemolysis in the lowest dilution (1 in 2); higher dilutions were completely negative. Further investigations of H.J.B.'s serum were made, and these are reported below.

With the exception of H.J.B. the only one showing any local reaction to the injections was W., in whom the first injection was followed by a delayed mild erythema while the second produced a slightly more severe reaction of the same type, but both were trivial in nature and quickly

TABLE I.—*Effect of Multiple Casoni Tests with Sheep Fluid on the Complement-fixation Reaction in Individuals Previously Sensitized Against Sheep Protein*

Volunteer and Date of Last Anti-rabies Injection	Reaction to Casoni Test	Time of Test Bleeds Related to Casoni Tests	Complement-fixation Reaction	
			Hydatid Antigen	Sheep-protein Antigen
W. (1927)	$\pm$ +	Before After 1st dose " 2nd "	Negative " "	Negative $\pm(2)$ ++(4)
L. (1938)	Negative "	Before After 1st dose " 2nd "	" " "	Negative " "
F. (1938)	Negative "	Before After 1st dose " 2nd "	" " "	" " "
P. (1939)	Negative "	Before After 1st dose " 2nd "	" " ?	+(2) ++(4)
H.J.B.* (1939)	Negative ++ +++	Before After 1st dose " 2nd " " 3rd "	Not done " ++(16)	Not done " ++(16)

Figures in parentheses = reciprocals of complement-fixation antibody titres. Controls: Individuals not previously sensitized with sheep vaccine gave completely negative results after two weekly intradermal injections of sheep hydatid fluid.

* H.J.B. was not test-bled before he received his first intradermal injection, but the fact that his sheep protein and hydatid antibody levels both fell gradually to zero within the following 12 months indicated that, prior to the experiment, his serum also did not contain detectable antibodies to either antigen. The results have therefore been incorporated in a single table.

faded. H.J.B., on the other hand, was quite severely affected. He had a strong familial and personal history of allergic manifestations, but the reactions to the intradermal injections were much greater than had been anticipated. The first injection into the left forearm produced little or no reaction; but the second, after a week's interval, into the same forearm, although there was no immediate reaction, was followed after a delay of five to six hours by an intense erythema spreading from the site of the injection to an area of about 15 cm. across. After a week, when this reaction had almost faded, the third injection was given into the opposite forearm. The injection weal quickly spread to nearly 10 mm., but without pseudopodia. Within four hours an acute erythema was rapidly extending from the site of injection and soon covered the whole of the forearm, which became markedly oedematous and heavy. There were also mild general symptoms. At the same time the other arm flared up at the site of the second injection with a more intense reaction than previously noted. It was more than three weeks before the arms resumed their normal appearance. Blood taken shortly after this and examined with different antigens gave the following results in the C.F.T.:

Standardized sheep hydatid antigen	Positive (titre 1 in 16)
*Worm scolices antigen (specific hydatid)	Positive (titre 1 in 16)
†Beef hydatid fluid	Weak positive (titre 1 in 2)
Normal sheep-brain antigen	Positive (titre 1 in 16)

A mass of washed scolices was added to about 2 ml. of H.J.B.'s serum, and after one hour on the bench was allowed to act at 4° C. overnight. The complement-fixing antibody titre with sheep hydatid antigen was reduced by this procedure from 1 in 16 to just under 1 in 4, but the titre to the sheep-brain antigen remained unchanged at 1 in 16. The

*Fresh scolices from hydatid cyst fluid, after thorough washing to remove host protein, were ground up in saline and the supernatant assayed against standard antibody. In the optimal dilution this antigen gave standard fixation with standard antibody at the correct titre and failed to show any cross-reaction with sheep-protein antibody; it was assumed to be specific for hydatid antibody.

†This beef hydatid fluid, when examined in parallel with the standardized sheep hydatid antigen in a chessboard test against the standard hydatid antibody, proved to be a weak antigen; at its optimal dilution there was standard fixation (++) at 1 in 32 instead of 1 in 64, and, moreover, the optimal dilution was only 1 in 5, whereas the sheep hydatid antigen had an optimal dilution of 1 in 16, which gave standard fixation at a titre of 1 in 64. Weak antigens are notoriously unreliable in immunity reactions, and there seems to be little doubt that the titre recorded in the test is too low.

sheep-brain antibody level was not reduced by absorption with guinea-pig kidney tissue, and absorption with sheep brain or sheep or ox red blood cells made the serum markedly anticomplementary. In spite of these difficulties it seems clear that H.J.B.'s serum contained two distinct antibody fractions—one specific against the hydatid antigen and the other against sheep protein.

Inoculation of Individuals with Anti-rabies Vaccine

Many unsuccessful attempts had been made to obtain samples of serum before and shortly after the completion of a course of injections of sheep-brain anti-rabies vaccine, but we were eventually able to examine a number of sera from patients in Hong Kong who had just completed a course of injections of goat-brain anti-rabies vaccine. Unfortunately the majority of the sera proved to be anticomplementary, but three sera were free from this effect, and the results of the C.F.T.s with these sera and hydatid antigens of ovine origin and a normal sheep-brain antigen are set out in Table II.

only, and in common with most other workers in this field we have relied on sheep fluids. By examining fluids from separate cysts it has been possible to select those very rich in antigen which can be accurately standardized and give reproducible results over periods of years. Their working dilution is generally 1 in 16 or 1 in 32: these high dilutions greatly reduce the chances of non-specific reactions. In our experience bovine fluids have not been so rich in hydatid antigen, and the results of the C.F.T. employing this antigen have been much less satisfactory.

When a rabbit serum containing high-titre natural sheep antibody was tested against sheep hydatid fluid and normal sheep-brain suspension in parallel it was found that the titre with the former was much less than with the latter. The sheep hydatid fluid had been assayed against the standard hydatid antibody for the hydatid C.F.T., and because of its richness in hydatid antibody it required dilution well beyond the optimum for maximum sensitivity for the measurement of sheep-protein antibody. The hydatid fluid, therefore, will react only at higher concentrations of sheep-protein anti-

TABLE II.—*Effect of Recent Sheep-protein Immunization on Complement Fixation with Different Antigens*

Serum	Complement-fixation Reaction—Serum Dilution Reciprocals													
	Sheep Hydatid Antigen							Normal Sheep-brain Antigen						
	2	4	8	16	32	64	128	2	4	8	16	32	64	128
Anti-rabies HK 4	+	+	±	—	—	—	—	+++	+++	+++	++	+	±	—
„ HK 5	±	±	—	—	—	—	—	+	+	±	±	—	±	—
„ HK 7	+++	+++	+++	+	±	—	—	+++	+++	+++	+++	++	+	—
Standard hydatid antibody ..	+++	+++	+++	+++	+++	++	±	—	—	—	—	—	—	—
„ sheep „	—	—	—	—	—	—	—	+++	+++	+++	+	—	—	—
Normal human serum ..	—	—	—	—	—	—	—	—	—	—	—	—	—	—

It is clear from this table that the sera of recently immunized individuals (with sheep- or goat-brain anti-rabies vaccines) may give positive complement-fixation reactions with a sheep hydatid antigen. Individual response to those inoculations, as judged by the results with the three specimens available, appears to vary considerably, and, as in the cases of the rabbit natural antibody, it seems that it is only when the sheep-protein antibody titre is high enough that there will be any demonstrable antibody titre with the sheep hydatid antigen.

Discussion

Although the hydatid C.F.T., employing sheep hydatid fluid as the antigen, has long been regarded as a specific test it is obvious that an antigen containing a factor derived from the host's tissues in addition to the specific component is liable to yield anomalous results under certain conditions. In view of the very large numbers of individuals (living in areas where rabies is endemic and hydatid disease not a great rarity) who have received the Semple type of anti-rabies vaccine it is surprising that results of the sort reported here have not been previously encountered. At least we have been unable to find any published records, although Kellaway and Fairley (1932) mentioned the possibility of previous sheep-protein sensitization interfering with the interpretation of the Casoni test but did not mention this complication in relation to the C.F.T. In spite of the fact that the test was introduced over 50 years ago (Ghedini, 1906) it seems likely that it is not employed very widely, otherwise the hazard would have been reported earlier.

From the limited evidence available induced sheep-protein antibody appears to fall to almost zero within 12 months, so that, provided no boosting injections are given in the interval, anti-rabies vaccine given a year or more previously would probably not complicate the interpretation of a hydatid C.F.T.

It is possible to prepare an almost completely specific hydatid antigen from fresh scolices as described briefly above, but the difficulty of obtaining suitable material in this country has restricted its use to experimental testing

body and fail altogether when the total amount is below a certain level. This is well illustrated in the case of HK 4 (see Table II, top line) and of the guinea-pig anti-sheep serum.

The serological results in the case of H.J.B. are clearly in a different category. The very intense tissue reactions to the intradermal injections of the sheep hydatid fluid in a sensitized allergic subject resulted in the development of true antibodies to both the hydatid and sheep factors to approximately the same titre. It is possible that Graña's (1944) observation that the injection of hydatid fluid into patients with hydatid disease resulted in the development of sheep antibodies, if the injections gave rise to tissue reactions, is an example of the same order but with a reversal of the sensitizing agent.

Summary

The occurrence of false-positive reactions in the routine hydatid complement-fixation test is described as a rare event, and evidence is produced to show how the use of an antigen containing both the host protein and the specific hydatid fractions can confuse the issue in patients previously immunized with vaccines containing the same or related host protein.

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