16. Newsholme, E. E., Randle, P. J., Manchester, K. L., Nature, 1962, v193, 370.

17. Shipp, J. C., Opie, L. H., Challoner, D. C., ibid., 1961, v189, 1018.

18. Evans, J. R., Opie, L. H., Renold, A. E., Am. J. Physiol., 1963, v205, 971.

19. Williamson, J. R., Glycolytic Control Mechanisms. J. Biol. Chem., 1965, v240, 2308.

20. Newsholme, E. A., Randle, P. J., Biochem. J., 1961, v80, 655.

21. Passonеau, J. V., Lowry, O. H., Biochem. & Biophys. Research Communs., 1963, v13, 372.

22. Parmeggiani, A., Bowman, R. H., ibid., 1963, v12, 268.

23. Garland, P. B., Randle, P. J., Newsholme, E. A., Nature, 1963, v200, 169.

24. Williamson, J. R., Jones, E. A., Azzone, G. F., Biochem. & Biophys. Research Communs., 1964, v17, 696.

25. Gimeno, A. L., Lacuara, J. L., Gimeno, M. F., Ceretti, E., Webb, J. L., Molecular Pharmacol., 1966, v2, 67.

26. Yang, W. C., Am. J. Physiol., 1963, v205, 781.

27. Busch, H., Martin, W. R., Nyhan, W. L., Zaratzian, V., Science, 1956, v124, 981.

28. Martin, W. R., Busch, H., Nyhan, W. L., Abdulian, D. H., J. Pharm. Exp. Therap., 1957, v119, 165.

29. Martin, W. R., Abdulian, D. H., Unna, K. R., Busch, H. A., ibid., 1958, v124, 64.

30. Peters, R. A., Sinclair, H. M., Thompson, R. H. S., Biochem. J., 1946, v40, 516.

31. Searls, R. L., Peters, J. M., Sanadi, D. R., J. Biol. Chem., 1961, v236, 2317.

32. Webb, J. L., Hollander, P. B., Circulation Res., 1959, v7, 131.

33. Opie, L. H., Evans, J. R., Shipp, J. C., Fed. Proc., 1961, v20, 302.

34. Lüttgau, H. C., Niedergerke, R., J. Physiol., 1958, v143, 486.

35. Webb, J. L., Hollander, P. B., personal communication.

36. ———, Circulation Res., 1956, v4, 618.

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Use of Xenogeneic (Heterologous) Sera for Cultivation of Rabbit Lymphocytes *in vitro*.* (31630)

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In vitro cultivation of the peripheral lymphocytes of many species, including the rabbit, usually requires media enriched with serum(1,2,3). Antisera to rabbit IgG or to whole rabbit serum prepared in other species can induce "blast transformation" of rabbit lymphocytes *in vitro,* while normal sera from these other species have very little or no blastogenic effect(3,4). In recent attempts to define better the induction of transformation with antisera, sheep were chosen for the preparation of more specific antisera to rabbit serum proteins, as sheep sera were found to be capable of supporting the viability of rabbit lymphocytes *in vitro* in our previous studies. However, normal sheep sera obtained from local sources were either toxic or did not satisfactorily maintain the viability of rabbit lymphocytes *in vitro*. This communication delineates the culture conditions under which the effect of the sheep antisera upon rabbit lymphocytes *in vitro* could be evaluated.

Materials and methods. Healthy adult rabbits with known immunoglobulin allotype were used as donors of lymphocytes for culture. Blood was obtained from the marginal ear vein and lymphocyte-rich suspensions were prepared as described previously, using 3% w/v pig skin gelatin(3). The cell concentration was determined by counting in a standard hemocytometer. The lymphocytes were washed once in Eagle's suspension medium (Microbiological Associates, Inc., Bethesda, Md.) containing 1/10th volume tryptose broth (Difco), 200 units/ml of penicillin, and 100 units/ml of streptomycin. The lymphocytes were suspended in the volume

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TABLE I. Effect of Normal Xenogeneic Sera on Rabbit Lymphocytes After 48 Hours *in vitro*.

	Whole serum		Whole serum + PHA*		Decomplemented serum		Decomplemented serum + PHA*	
Serum source	Viability	% Blast transformation	Viability	% Blast transformation	Viability	% Blast transformation	Viability	% Blast transformation
Rabbit	good	<1	good	83	good	<1	good	68
Newborn calf	fair	<1	toxic		fair	3	toxic	
Sheep	toxic		"		poor	3	"	
Calf	good	2	good	75	good	<1	good	54
Fetal bovine	"	3	"	60	"	1	"	49
Monkey	poor		toxic		fair	5	toxic	
Lamb	toxic		"		"	2	"	
Human	poor		"		—	—	—	—
Bovine	toxic		"		—	—	—	—
Horse	"		"		—	—	—	—

* PHA—0.03 ml phytohemagglutinin added to each culture bottle.

of medium necessary to give a concentration of 5×10^6 lymphocytes per ml. One ml of this suspension was added to 2 ml of medium in bijou bottles. A volume of 0.5 ml of the appropriate serum was added to each culture. Three hundredths ml of phytohemagglutinin were also added to some cultures(1), (Difco, Bacto, phytohemagglutinin M., Difco Laboratories, Detroit, Mich., lot 0528). After 48 hours each culture was transferred to a centrifuge tube, the cells present obtained by centrifugation at 1000 rpm for 10 minutes. Smears were made from these cells on glass slides. The smears were stained with Jenner-Giemsa at pH 5.5(5) and the degree of toxicity or transformation was estimated morphologically.

Horse (lot no. 43413), monkey (59420), human (59612), lamb (56732), bovine (58713), calf (58463, 59169), newborn calf (56488) and fetal bovine (59713) sera were obtained commercially (Microbiological Associates). Sheep sera were procured locally (Mr. John Lawrence, Hartwood Farm) by sterile bleeding from the jugular vein, and rabbit sera were obtained from the supernatants of the lymphocyte preparations. Decomplementation was accomplished by heating the sera at 56°C for 30 minutes. Fractionation of 3-4 ml samples of sera on Sephadex G-200 was accomplished on 2.5×100 cm columns with 0.2 M tris-buffered saline at pH 8.0 using reverse flow. The fractions were monitored by recording the optical density at 254 mμ by an LKB Model 4701A unicord. The first (19S), second (7S) and third (3-4S) peaks obtained were concentrated by ultrafiltration and tested for reactivity with rabbit cells by hemagglutination and hemolysis. Human IgG was obtained from the 0.01 M phosphate buffer pH 8.0 eluate of human serum fractionated on diethylaminoethyl cellulose. Hemagglutination and hemolysis titrations were performed by adding one drop of 1% normal rabbit erythrocytes (washed and suspended in phosphate buffered 0.85% NaCl, pH 7.2) to the sera dilutions, using the microtitration procedure of Takatsy (6) as modified by Sever(7). The plates were incubated for 1 hour at room temperature, then overnight at 4°C. The total serum protein was determined refractometrically(8) and the electrophoretic distribution of serum protein determined using cellulose acetate electrophoresis(9).

Results. The results obtained when rabbit lymphocytes were cultured using the normal sera of different species to enrich the culture medium are shown in Table I and in Fig. 1. Of the 10 different sera used, only rabbit, calf and fetal bovine sera gave good results. The addition of any of the other sera to lymphocyte cultures resulted in death of the lymphocytes and lysis of the erythrocytes present or in unsatisfactory viability of the lymphocytes. After decomplementation the toxic sera no longer caused death of all the cells, but marked agglutination of both erythrocytes and lymphocytes was observed and viability, as judged by histologic appearance, was unsatisfactory (Fig. 1C). When phytohemagglutinin was added, transformation of

the rabbit lymphocytes *in vitro* was supported only by rabbit, calf and fetal bovine serum (Fig. 2). Further observations were made with normal sheep sera as this was the system of greatest interest in the present study. The hemagglutinating properties of the normal sheep sera could be removed by absorption of the decomplemented sheep sera with 1/3 volume packed, washed (phosphate burfered 0.85% NaCl pH 7.2) rabbit erythrocytes overnight at 4°C. Such absorbed sera, however, still did not satisfactorily support the viability of the rabbit lymphocytes *in vitro* (Figfl 1 D). However, good survival could be supported by a combination of one-half ml of calf or rabbit sera and one-half ml of normal sheep serum (Table II, Fig. 2 A). The effect of ½ ml of a potent sheep antiserum to rabbit IgG using this culture system is shown in Table II and Fig. 2 D. Addition of the sheep antisera to rabbit IgG stim-

TABLE II. Effect of Mixtures of Sera on Rabbit Lymphocytes After 48 Hours *in vitro*.

Serum source*	% Blasts	Viability
Allogeneic (rabbit)	<1	good
Calf	<2	"
Normal sheep A†	—	toxic
Calf & normal sheep A	<1	good
Calf & sheep anti-IgG A	68%	"

* 0.5 ml of each serum added to each culture bottle.

† A = absorbed with rabbit erythrocytes.

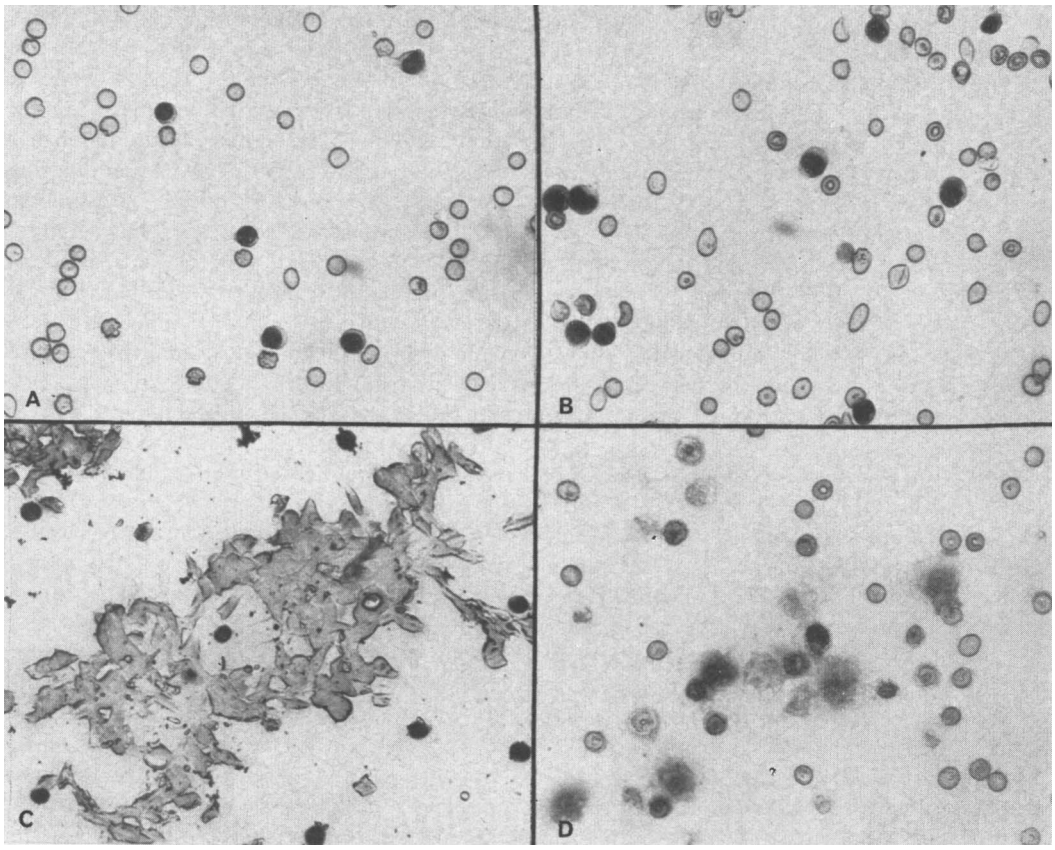

FIG. 1. Rabbit peripheral lymphocytes cultivated for 48 hr in medium enriched with: A, rabbit serum; B, calf serum; C, decomplemented sheep serum; D, decomplemented normal sheep serum absorbed with rabbit erythrocytes. Differences in size between normal small lymphocytes seen in A and in B are similar to the variation seen in different areas of the same smear. Decomplemented sheep serum causes marked agglutination and lymphocyte viability is poor (C). This agglutination may be removed by absorption with rabbit erythrocytes, but such treated sheep sera do not maintain lymphocyte viability. One viable lymphocyte is present in D, along with the remains of dead lymphocytes. Magnification, 250 ×; Enlargement 1½ ×.

ulated a high percentage of blast transformation (60-80%) that was reproducible in 3 separate experiments.

In an attempt to delineate partially the toxic factor present in the xenogeneic sera, the hemolytic and hemagglutinating capacity of the whole sera, decomplemented sera and the concentrated peaks of Sephadex fractions of whole sera were tested. All of the toxic sera contained hemolysing or agglutinating factors which were present in the first (19S) and second (7S) but not the third (3-4S) peaks following Sephadex fractionation (Table III). Rabbit, calf and fetal bovine sera, which were not toxic for rabbit lymphocytes *in vitro,* did not contain lytic or agglutinating factors. Human IgG prepared by diethylaminoethyl cellulose chromatography contained significant hemagglutinating activity.

TABLE III. Log 2 Hemagglutination or Lysis Titers of Sera and Sephadex Fractions of Sera Used for Cultivation of Rabbit Lymphocytes *in vitro.*

Serum source	Whole serum	Decomplemented serum	Sephadex fraction (1)	(2)	(3)
Rabbit	0	0	0	0	0
Sheep	3*	3	5*	3	0
Calf	0	0	0	0	0
Fetal bovine	0	0	0	0	0
Newborn calf	1	±	0	1	0
Bovine	2	1	1	1	0
Lamb	3*	1	3	4*	±
Human	6*	4	5	2	0
Monkey	6*	3	3	—	—
Horse	3*	2	2*	2	0

* Lysis.

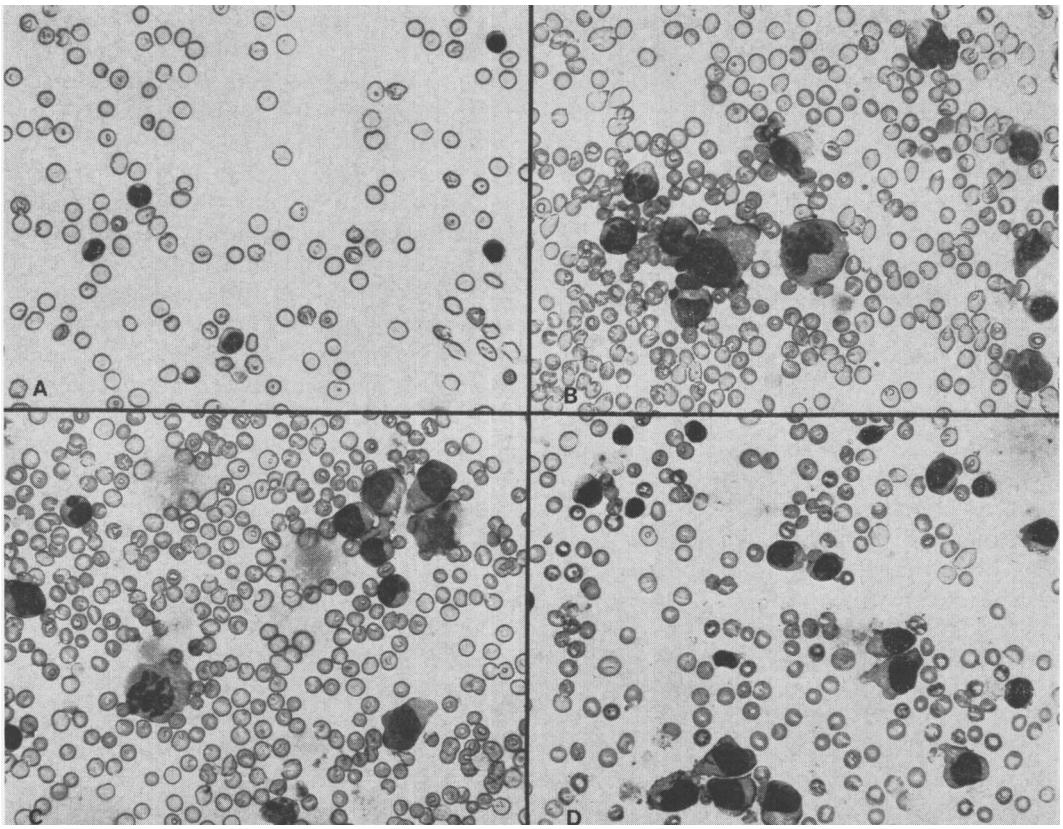

FIG. 2. Rabbit peripheral lymphocytes cultured for 48 hr in medium enriched with: A, calf serum and decomplemented absorbed normal sheep serum; B, rabbit serum and phytohemagglutinin; C, calf serum and phytohemagglutinin; D, calf serum and decomplemented, absorbed sheep antiserum to rabbit IgG. Typical blast cells are present in B, C, and D, and a mitotic figure is shown in C. Much more agglutination is generally present in the smears from cultures to which phytohemagglutinin has been added, but the above areas were chosen in order to better demonstrate the cellular morphology. Magnification 250 ×; Enlargement 1½ ×.

TABLE IV. Protein Distribution of Sera Used for Cultivation of Rabbit Lymphocytes, g %.

Serum source	Total	Albumin	α	β	γ
Fetal bovine	4.1	2.6	1.1	.3	.1
Newborn calf	5.8	2.5	1.0	1.3	1.0
Calf	4.1	2.5	1.1	.4	.1
Bovine	8.1	3.3	1.2	1.6	2.0
Lamb	5.7	3.4	.4	.8	1.1
Monkey	5.5	2.9	.5	1.3	.8
Human	6.6	3.7	.8	.8	1.3
Sheep	6.3	3.3	.9	.8	1.3
Rabbit	6.0	3.0	1.1	.9	1.0

Estimation of the protein fractions of the sera used revealed that all of the sera except calf and fetal bovine sera contained > 0.8 g% of γ-globulin (Table IV). Calf and fetal bovine sera with less than 0.1 g% γ-globulin were the only xenogeneic sera that were not toxic for rabbit lymphocytes. Rabbit sera (allogeneic) contained 1.0 g% γ-globulin but were not toxic for rabbit lymphocytes.

Discussion. Although some mammalian cells may be grown in a completely synthetic medium without serum(10,11), *in vitro* cultivation of mammalian lymphatic tissue generally has required enrichment of the culture medium with 5-20% of whole serum(12,13). However, reports of the successful culture of lymphatic tissue(10,14) and peripheral leukocytes(15) in serum free culture medium have been published. Attempts to maintain the viability of the peripheral lymphocytes of rabbits *in vitro* in the absence of serum have been unsuccessful in this laboratory. In addition, the enrichment of the lymphocyte cultures with the sera from many xenogeneic (heterologous) species including human, monkey, horse, sheep, lamb, bovine and newborn calf resulted in death of most of the lymphocytes and lysis of the erythrocytes present. Analysis of these xenogeneic sera indicated that they contain a naturally occurring toxic factor for rabbit blood cells. Toxicity was not present in fetal bovine and calf sera with low γ-globulin levels and was also not present in allogeneic rabbit sera with normal γ-globulin levels, suggesting that the toxicity is due to naturally occurring antibodies.

The cytotoxicity of nweborn calf and bovine sera, in view of the lack of toxicity in fetal calf and calf sera, may be explained by the serum γ-globulin concentration. There is little or no placental transfer of γ-globulin in the cow so that no antibody is found in the blood of fetal calves from immune mothers(16,17). The newborn calf rapidly absorbs antibodies from colostrum and after suckling, a newborn calf will have high titers of antibody in the blood(17,18). This level falls off exponentially when suckling ceases and the calf does not begin to make significant γ-globulin of its own until later in life. Thus, calf and fetal bovine sera contain very little or no γ-globulin while newborn calf and adult bovine sera have considerably higher serum γ-globulin levels.

The main purpose for this study was to establish a technique to evaluate the activity of sheep antisera to rabbit serum protein on rabbit lymphocytes *in vitro*. This effect could not be determined directly, since normal sheep sera were toxic for rabbit lymphocytes *in vitro*. Good results were obtained when the cultures were supplemented with 15% calf or rabbit serum prior to addition of normal sheep sera. The effect of sheep antisera to rabbit serum proteins could not be studied using rabbit serum in the culture as the antibody activity would be absorbed by reacting with the rabbit serum proteins. Accordingly, rabbit lymphocyte cultures were set up with mixtures of normal calf serum and the appropriate sheep antiserum. This resulted in satisfactory experimental results as mixtures of decomplemented normal sheep serum absorbed with rabbit erythrocytes and calf serum resulted in histologically good lymphocytes with less than 3% blasts present after 48 hours in culture. Addition of decomplemented sheep antisera to rabbit IgG absorbed with rabbit erythrocytes to the lymphocyte cultures containing calf serum produced as high as 60-80% blast cells.

The increase in background blast transformation from < 1% with rabbit sera to up to 5% with some xenogeneic sera is consistent with the report of Schrek and Elrod(19) that addition of rabbit sera to cultures of rat and human lymphocytes produces a small number of transformed cells after 2 to 4 days *in vitro*. However, others have been unable to find any transformation of human lymphocytes cultured in rabbit(20-22) or calf(23)

serum. The variation in results is most likely due to the very low rate of transformation with the xenogeneic sera. Such transformation probably depends upon the species of lymphocytes cultured and upon the sera used. The high rate of spontaneous blast transformation of guinea pig and rabbit lymphocytes as reported by Sabesin(24) has not been found in any other laboratory, although we previously reported high rates of spontaneous transformation with one strain of monkey(2). The low level of transformation with normal calf-sheep serum mixtures permits the use of the calf and sheep sera supplemented media to study the effect of sheep antisera on rabbit lymphocytes *in vitro*. An explanation for the presence of toxic factors in the xenogeneic sera used for cultivation of rabbit lymphocytes *in vitro* in the current study in view of the lack of toxicity in previous studies(3,4) is not obvious at this time.

Summary. Normal xenogeneic (heterologous) sera from adult animals are toxic or do not support the growth of the peripheral lymphocyte of rabbits *in vitro*. Fetal bovine or calf sera are able to maintain rabbit lymphocytes satisfactorily in culture. The effects of antisera to rabbit serum proteins produced in adult sheep on rabbit lymphocytes *in vitro* can be studied using cultures enriched with calf serum. The sheep sera must be decomplemented and absorbed with rabbit lymphocytes prior to the addition of these sera to lymphocyte cultures.

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1. Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., Hungerford, D. A., Exp. Cell. Res., 1960, v20, 613.

2. Knight S., Ling N. R., Sell, S., Oxnard, C. E., Immunol., 1965, v9, 565.

3. Sell, S., Gell, P. G. H., J. Exp. Med., 1965, v122, 423.

4. Sell, S., Rowe, D. S., Gell, P. G. H., ibid., 1965, v122, 823.

5. Ling, N. R., Spicer, E., James, K., Williamson, N., Brit. J. Haemattol., 1965, v11, 421.

6. Takatsy, G., Acta, Micro. Acad. Sci. Hung., 1955, v3, 191.

7. Sever, J. L., J. Immunol., 1962, v88, 320.

8. Khanderia, S., Batsakis, J. G., Briere, R. O., Capps, R. D., Univ. Michigan Med. Bull., 1963, v29, 305.

9. Briere, R. O., Mull, J. D., Am. J. Clin. Path., 1964, v42, 547.

10. Trowell, O. A., Exp. Cell. Res., 1959, v16, 118.

11. Ham, R. G., Proc. Nat. Acad. Sci., 1965, v53, 288.

12. Makela, O., Suurmaa, S., Vainio, T., Ann. Med. Exp. Feun., 1964, v42, 57.

13. Dresser, A. M., Immunology, 1965, v9, 483.

14. Ambrose, C. T., J. Exp. Med., 1964, v119, 1027.

15. Hutton, W. E., Watson, E. D., Proc. Soc. Exp. Biol. and Med., 1964, v116, 924.

16. Orcutt, M. L., Howe, P. E., J. Exp. Med., 1922, v36, 291.

17. Brambell, F. W. R., Biol. Rev., 1958, v33, 448.

18. McDiarmid, A., Vet. Rec., 1946, v58, 146.

19. Schrek, R., Elrod, L. M., Lancet, 1964, vii, 595.

20. Grasbeck, R., Nordman, C. T., Chapelle, A., Acta, Med. Scand., 1964, Supp. 412, 39.

21. Byrd, W. S., Finley, W. H., Finley, S. C., McLure, S., Lancet, 1964, v2, 420.

22. Marshall, W. H., Norins, L. C., Australian J. Exp. Biol. Med., Sci., 1965, v43, 213.

23. Oppenheim, J. J., Whang, J., Frei, E., J. Exp. Med., 1965, v122, 651.

24. Sabesin, S., Science, 1965, v149, 1385.

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