Table 1. Intracellular stages of *Sarcocystis* observed in various types of cultured cells; × indicates presence of intracellular stage listed.

Cell type	Serial passage	Experiments (No.)	Leighton tubes per experiment (No.)	Organisms inoculated per tube (No. × 1000)	Intracellular stages				
					Uninucleate			Multinucleate	Cyst-like
					Banana-shaped	Ellipsoid	Round		
EBK	25	3	7	300, 500, 1600	×	×	×	×	×
EBTr	36	3	7	300, 500, 500	×	×	×		×
MDCK	52	3	7	300, 500, 1600	×	×			
ECK	Primary	2	7	3000	×	×	×	×	
ECM	Primary	2	7	3000					
ETK	Primary	1	6	200	×	×	×	×	

were large ellipsoidal or oblong bodies. Twenty of these in ECK cells averaged 3.8 by 7.2 μm. Changes in the parasite nucleus were observed before and after this transformation process in specimens fixed at 3, 6, 12, and 24 hours (Fig. 1). These changes are similar to those described for the transformation of *Eimeria bovis* sporozoites into trophozoites (*4*). In both stained and live specimens (Fig. 2B), the cytoplasm of the ellipsoid-oblong parasite contained many small granules not observed in other stages. Some living parasites rapidly rotated clockwise on their longitudinal axis, stopped, and rotated counterclockwise. This sequence was repeated several times.

At 30 hours, intracellular organisms in EBK, EBTr, ECK, and ETK cell cultures were nearly round but otherwise appeared very similar to the ellipsoid-oblong form. Twenty organisms in ECK cells averaged 5.2 by 7.2 μm. Living specimens rotated clockwise and counterclockwise for several rotations in each direction or alternatively reversed directions, moving through an arc of 90°.

An organism undergoing nuclear division was observed in EBTr cell cultures at 48 hours (Fig. 2C). Two- to nine-nucleate stages, resembling young eimerian schizonts, were found as early as 30 hours in ECK cells and in EBK, ECK, and ETK cells at 48 and 72 hours (Fig. 2D). Twenty such forms in 48-hour ECK cells averaged 6.5 by 7.5 μm.

Intracellular bodies observed in EBK and EBTr cell cultures at 48 and 72 hours were bounded by a thick wall which imparted a cyst-like appearance. Twenty such forms averaged 6.5 by 8.5 μm. Most of these cyst-like bodies were ovoid or lemon-shaped. A sphere of what appeared to be granular cytoplasm occupied more than half the volume and was located toward one end of the body (Fig. 2E). A less dense material was present between the sphere and the other end of the body. A few bodies contained two inclusions, some resembling the banana-shaped organisms (Fig. 2F).

Although caution must be exercised in interpretation, it is interesting to note that the cell types used so successfully for the cultivation of eimerian species (*5*), which parasitize the intestinal tract, have also supported development of *Sarcocystis*, and that *Sarcocystis*, which has been found only in muscle tissue, did not even enter cultured muscle cells. Recent studies on *Toxoplasma gondii* (*6*) indicate that this parasite develops, in the intestine of cats, through stages identical with those of coccidian parasites. The possibility that intestinal stages occur in the life cycle of *Sarcocystis* has long been suspected on the basis of studies on infection by fecal contamination (*1*). The finding of nuclear and cytoplasmic transformation as well as multinucleate stages similar to those in *Eimeria* further suggests that *Sarcocystis* may be closely related to this genus. The significance of the cyst-like bodies is not known. Their appearance is similar to the oocyst stage of *Eimeria*, but they are much smaller and identification of preceding sexual stages, as in oocyst development, has not been concluded.

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Lymphocytic Responses to Streptococcal Antigens in Glomerulonephritic Patients

Abstract. *The lymphocytes from patients with progressive glomerulonephritis showed significant inhibition of cell migration in the presence of group A streptococcal particulate antigens. Marked increases in the level of DNA synthesis of these lymphocytes were also observed after contact with these antigens. Lymphocytes from patients with unrelated renal disorders exhibited minimum reactivity to streptococcal antigens.*

The exact nature of the pathological mechanisms involved in progressive human glomerulonephritis remains obscure (*1*). While studies in both experimentally induced glomerulonephritis and acute post-streptococcal nephritis in man suggest that antigen-antibody complexes are mediators of the renal damage (*2–5*), evidence of the continuing participation of complexes in progressive nephritis are more difficult to obtain. In experimentally induced progressive glomerulonephritis there is a gradual attrition of glomerular-deposited, immune complexes after cessation of administration of the antigens (*6*) and immunofluorescent studies with streptococcal antiserums of renal biopsy specimens in man have failed to demonstrate streptococcal products in the renal lesions in these patients (*4*, *7*). In one of these studies (*4*), fluorescein-labeled streptococcal antigens did bind to areas of "fixed" gamma

Table 1. The effect of streptococcal antigens on DNA synthesis, as measured by [C^{14}]thymidine incorporation, in lymphocytes from normal and glomerulonephritic subjects. Each case tested was the mean of three parallel tubes.

Substance tested	Nonnephritic patients		Glomerulonephritic patients	
	No.	Mean count/min (± S.E.)	No.	Mean count/min (± S.E.)
Normal mediums	7	88 (± 20)	14	66 (± 13)
T12 Streptococcal membrane 0.1 mg/ml	7	832 (± 229)	14	2144 (± 223)
0.05 mg/ml	5	472 (± 161)	8	1768 (± 296)
0.01 mg/ml	5	377 (± 117)	8	1284 (± 349)
T12 Streptococcal cell wall 0.1 mg/ml	7	170 (± 96)	14	1079 (± 265)
0.05 mg/ml	5	50 (± 3)	8	707 (± 235)
0.01 mg/ml	5	59 (± 7)	6	207 (± 83)
T5 Streptococcal membrane 0.1 mg/ml	2	759 (± 123)	5	1199 (± 215)
T5 Streptococcal soluble cell wall 0.1 mg/ml	6	47 (± 5)	2	91 (± 6)

globulin in the glomeruli, but the partially purified streptococcal preparation used in this study contained streptococcal membrane fragments. In view of the known immunological cross-reactivity between group A streptococcal membranes and human glomerular basement membranes (*8*), the specificity of the binding of the streptococcal preparation to fixed antibody remains open to question.

These studies suggested the possibility that altered cellular reactivity to either streptococcal antigens or human glomerular basement membranes, or both, might play a role in the pathological processes responsible for the continuing disease process in progressive glomerulonephritis. This hypothesis was further strengthened by both the recent finding of Bendixen that the peripheral lymphocytes of patients with progressive glomerulonephritis are specifically sensitized to fetal renal tissue homogenates (*9*) and at least two reports of the production of progressive glomerulonephritis in rats by use of lymphoid cells from nephritis donor animals (*10*).

Using the technique of inhibition of cell migration and DNA synthesis as measured by [C^{14}]thymidine incorporation, we demonstrate in this report that the lymphocytes of patients with progressive glomerulonephritis are sensitized to particulate streptococcal antigens. Lymphocytes from patients with unrelated renal disorders failed to respond to these antigens.

Patients used in this study were all from the Renal Clinic of St. Eric's Hospital in Stockholm. The diagnosis of progressive glomerulonephritis was established by both laboratory and clinical parameters of the disease, as well as by immunofluorescent and histological examination of renal biopsy specimens. Patients with unrelated renal disorders, such as pyelonephritis and polycystic kidneys, served as controls.

The isolation of lymphocytes from these patients, lysis of contaminating red blood cells, and general preparation of lymphocytes for capillary migration studies were based on the procedures used by Möller and his associates (*11*). The cell counts were adjusted to a concentration of 2×10^7 cells per milliliter; cells were placed in capillary tubes and allowed to migrate either with or without the different antigens tested. The techniques utilized for the amount of migration and inhibition by antigen have been described (*12*). Earle's balanced salt solution containing added vitamins and amino acids was the medium used for all experiments. Twenty percent AB human serum obtained from normal volunteers was added to the medium.

For the DNA synthesis studies, human lymphocytes were cultivated as previously described (*13*). The cell count was adjusted so that 10^6 lymphocytes in 1 ml of the described medium were cultivated in tissue culture tubes with or without the addition of streptococcal antigens. Phytohemagglutinin (PHA; Burroughs Wellcome Ltd.) and purified protein derivative (PPD; State Bacteriological Institute, Copenhagen) were also used in certain experiments. The DNA synthesis induced by the various antigens or PHA was determined by adding 0.1 μc of [C^{14}]thymidine (Amersham, England) for 24 hours to each tube. Isotope was added to PHA tubes 48 hours after the initiation of the culture, while the optimum time for the addition of [C^{14}]thymidine to the other tubes was found to be 96 hours. Twenty-four hours after the addition of [C^{14}]thymidine, the cells were washed twice and 1 ml of formic acid was added to the pellet of packed cells. When the cells had dissolved, 0.3 ml of this solution was placed on a plastic planchette, and, after evaporation of the formic acid, they were counted on a gas flow Geiger detector (Nuclear-Chicago).

Group A streptococcal strains T12/126/1 (type 12) and A964 (type 5) were obtained from the Rockefeller University collection of Dr. Lancefield and were isolated from patients with post-streptococcal glomerulonephritis and acute rheumatic fever, respectively. Methods for the preparation of streptococcal membranes, cell walls, and soluble streptococcal structures have been described in detail (*14*). Lyophilized preparations of each material were used in all experiments.

Heat-killed tubercle bacilli (cultured and prepared by the State Bacteriological Institute, Stockholm) were used as an antigen at concentrations of 1 or 2×10^6 bacilli per milliliter of medium with lymphocytes from tuberculin-sensitized subjects (*15*) with or without glomerulonephritis.

The results of the cellular migration of lymphocytes from 13 patients with progressive glomerulonephritis and 13 patients with various nonnephritic renal disorders are summarized in Fig. 1. The addition of 0.1 mg of highly purified group A streptococcal membranes or cell wall to the culture medium in which the lymphocytes migrated resulted in a marked degree of inhibition of the lymphocytes from all but one of the nephritic patients. The degree of migration inhibition by type 5 streptococcal membranes was somewhat less, but still highly significant. The reaction with the streptococcal cell walls appeared at first to be paradoxical. However, it is known that these walls contain at least 30 percent (by weight) of membrane contamination, as determined by the amount of glucose present in the cell wall preparation (*14*). In contrast, soluble streptococcal preparations, including the group-specific carbohydrate, mucopeptide, surface antigens (M and T proteins), cytoplasmic materials, and bacterial nucleic acids were not effective in inhibiting the migration of nephritic lymphocytes. Only minimum degrees of inhibition to streptococcal antigens were observed in the lymphocytes of 13 nonnephritic con-

trol patients. The observation that the degree of lymphocytic inhibition to tubercle bacilli was similar in all patients indicates that lymphocytes from patients with progressive glomerulonephritis do not respond abnormally to any antigenic stimulus.

The evidence that streptococcal membranes and cell walls inhibited lymphocyte migration in patients with progressive glomerulonephritis indicated an altered cellular reactivity to streptococcal antigens. In order to explore this altered reactivity further, another parameter of cellular hypersensitivity was employed. The level of DNA synthesis in lymphocytes, as measured by the incorporation of [C^{14}]-thymidine following exposure to a sensitizing antigen, is another measurement of cellular reactivity to antigenic confrontation (*16*). While this reaction measures the response of both antigen-sensitive cells and antibody-producing cells, it is considered a reliable in vitro parameter of cellular reactivity to antigenic stimulation. In addition, this technique has the advantage of longer contact with the antigen (4 to 5 days versus 24 hours) and measures lymphocytic reaction to soluble and particulate antigens.

The data in Table 1 show the markedly increased DNA synthesis induced in the lymphocytes of patients with chronic glomerulonephritis after cultivation with streptococcal antigens. It is apparent that both streptococcal membranes (T12 and A964), as well as the cell walls from the type 12 strain, produced four to ten times the amount of [C^{14}]thymidine incorporation in the lymphocytes of nephritic patients as that observed in the lymphocytes of nonnephritic controls. The fact that the nonnephritic patients also responded to the streptococcal antigens is again not surprising, since the general population has had at least minimum exposure to streptococcal infections. However, the reactivity of the lymphocytes of these patients to the streptococcal antigens was always much less than that observed with lymphocytes from patients with progressive glomerulonephritis. While the reactivity to streptococcal cell walls was always less than that observed with membranes in nonnephritic controls, the response of lymphocytes from nephritic patients to streptococcal cell walls was as much or more than that observed with streptococcal membranes. Since altered cellular reactivity to streptococcal membranes (which contain negligible amounts of streptococcal cell walls) was quite pronounced in the lymphocytes of glomerulonephritic patients, these results suggest that the antigenic configuration of the sensitizing antigen (presumably contaminating membrane fragments) was also important for the observed stimulation by the streptococcal cell walls. Again, soluble streptococcal preparations produced only minimum degrees of lymphocytic stimulation in nephritic and nonnephritic patients.

Table 2. The effect of PHA and PPD on DNA synthesis, as measured by [C^{14}]thymidine incorporation, in lymphocytes from normal and glomerulonephritic subjects. Each case tested was the mean of three parallel tubes.

Substance tested	Nonnephritic patients		Glomerulonephritic patients	
	No.	Mean count/min (± S.E.)	No.	Mean count/min (± S.E.)
Normal mediums	7	88 (± 20)	14	65 (± 13)
PHA (1/100)	7	1509 (± 188)	14	1583 (± 139)
PPD (0.01 mg/ml)	6	762 (± 236)	6	744 (± 221)
PPD (0.02 mg/ml)	6	783 (± 203)	4	689 (± 184)

The evidence that the increased reactivity of the lymphocytes from the patients with glomerulonephritis was specific for streptococcal antigens and was not merely a reflection of a general altered cellular reaction is seen in Table 2. The increase in DNA synthesis induced by PHA and PPD was similar in both groups of patients.

The present study adds another dimension to the concept of a cellular hypersensitivity reaction in patients with progressive glomerulonephritis. The degree of inhibition of lymphocyte migration achieved when purified streptococcal membranes were introduced into the culture medium for lymphocytes indicates that patients with chronic glomerulonephritis are specifically sensitized to streptococcal antigens—in particular, to the streptococcal membrane. The fact that streptococcal membranes from both a nephritogenic and a nonnephritogenic strain gave essentially similar results is at first surprising in view of the small number of streptococcal types capable of inducing glomerulonephritis (*1*). However, it is conceivable that all streptococcal strains possess the nephritogenic characteristics and only the ease with which nephritogenic antigens or toxins are exposed in vivo separates the "nephritogenic" and "nonnephritogenic" strains. The exposure of both membranes in this study might explain the similar results obtained with a nephritogenic (T12/126) and a nonnephritogenic (A964) strain. In contrast, the control nonnephritic group failed to respond to any of the streptococcal antigen preparations, indicating the specific nature of the altered state of lymphocytes in glomerulonephritic patients.

In turning to the exact role sensitized lymphocytes may play in progressive glomerulonephritis, two interpretations of the results reported above are possible. In view of the recent evidence for participation of glomerular basement membrane antigens in progressive human glomerulonephritis (*17*), perhaps

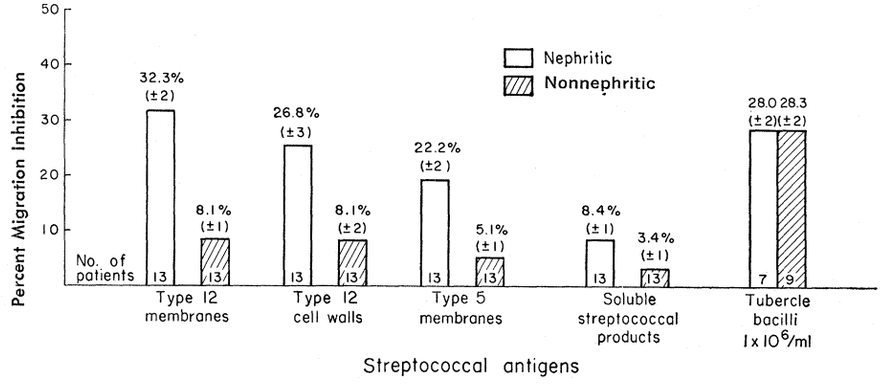

Fig. 1. The percentage of inhibition of lymphocytic migration with various streptococcal antigens and tubercle bacilli in patients with either progressive glomerulonephritis or unrelated renal disorders. The figures in parentheses refer to the standard error for each group.

the most plausible explanation of the observed cellular reactivity of lymphocytes from glomerulonephritic patients is primary sensitization to the glomerular antigens. The observed reactivity to the streptococcal membranes would be merely a reflection of the known shared antigenicity between the two membranes. This type of cross-reactive sensitization might also explain the observed exacerbations in progressive glomerulonephritis. However, in view of the paucity of information regarding the nature of some of the immunizing antigens in progressive glomerulonephritis (*18*), it is conceivable that sensitization to the strepococcal membrane antigens might be the initiating factor in the glomerular damage. Continued release of . glomerular . membrane . antigens coupled with intercurrent streptococcal infections could be responsible for the progressive nature of the disease. A closer correlation between the nature and deposition of glomerular antibodies and the cellular reactivity to human glomerular membranes and streptococcal membranes in acute and progressive glomerulonephritis, as well as more precise characterization of these antibodies with respect to the antigens, will be needed to clearly separate the two hypotheses.

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Bimolecular (Black) Lipid Membranes: Study of Lipid-Protein Interactions

Abstract. *An experimental method has been devised for the study of the interaction of bimolecular (black) lipid membrane and protein in which 8-anilino-1-naphthalenesulfonic acid is used as a fluorescent probe. The presence of phospholipid in the membrane is necessary for the enhanced fluorescence.*

The properties and functions of cellular membranes undoubtedly are based upon the interaction of their lipids and proteins. Although lipid-protein interaction has been widely discussed, little information is available from direct examination of cellular membranes. Accordingly, we report here some qualitative observations of lipid-protein interactions in a bimolecular lipid membrane (BLM) system in which 8-anilino-1-naphthalenesulfonic acid (ANS) was used as a fluorescent probe.

This acid and its derivatives have been used in studies of changes in protein conformations and in studies of interactions (*1*), because the fluorescence properties of ANS depend upon the polarity of its environment (*2*). There is almost no emission when the acid is dissolved in water (quantum yield is 0.004) but it becomes fluorescent when stoichiometrically bound to specific sites in the nonpolar regions of the proteins. An enhancement of fluorescence accompanies the binding of ANS to albumin, an indication that in solution the nonpolar portions of this protein are available to ANS penetration. Recently ANS has been used in studies on biological membranes (*3*).

Black lipid membranes (*4*) are formed from solutions of (i) oxidized cholesterol prepared by bubbling oxygen through a 4 percent solution of cholesterol in *n*-octane at its refluxing temperature (*5*); or (ii) a mixture of oxidized cholesterol ($10^{-2}M$) and egg lecithin ($10^{-3}M$). All reagents (*6*) were C.P. grade and were used without further purification.

The experimental setup (Fig. 1) was modified from the one used for measurement of BLM thicknesses (*5*). A 100-watt low-pressure mercury lamp with a selected line filter provided excitation at 365 nm. The BLM was viewed with a low-power microscope provided with a side arm to accommodate a photomultiplier tube (EMI 9558Q). The output from the photomultiplier tube

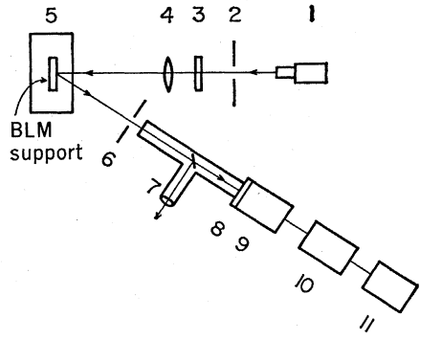

Fig. 1. Schematic diagram of the experimental setup used for studies of BLM-protein interaction; *1*, light source; *2*, slit; *3*, interference filter; *4*, condensing lens; *5*, cell assembly; *6*, slit; *7*, microscope and viewing tube; *8*, iris diaphragm; *9*, photomultiplier tube; *10*, microphotometer; *11*, recorder.

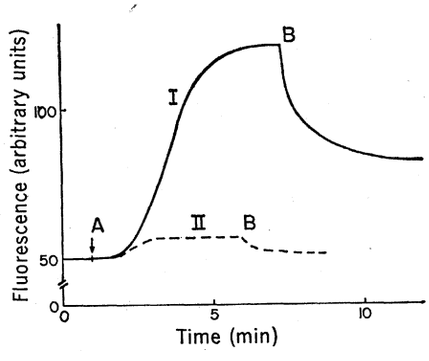

Fig. 2. Fluorescence of BLM treated with 8-anilino-1-naphthalenesulfonic acid and bovine serum albumin.