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Phagocytosis of Peripheral Nerve Myelin In Vitro: Effect of Antibody

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We have previously shown that antisera to whole CNS myelin, whole PNS myelin, galactocerebroside (GC), and myelin basic protein (MBP) promote the uptake of CNS myelin by cultured macrophages, and stimulate the conversion of myelin lipids to cholesterol ester and triglycerides. Here we report the results of similar studies using PNS myelin purified from the rat sciatic nerve. Antisera to whole CNS myelin, whole PNS myelin, GC, and MBP preincubated with ^{14}C-labeled PNS myelin increased the production of radioactive cholesterol ester by macrophages in culture to a level about twice that with preimmune serum, and five to six times that of untreated myelin. The amounts of [^{14}C]triglyceride were similarly increased with these antisera, whole P_0 and P_2 antisera had little or no effect. IgG prepared from the antisera stimulated lipid metabolism to almost the same extent, while heating the antisera did not decrease the stimulatory effect, indicating that myelin was opsonized by IgG, but not likely by complement. With a few exceptions, the four active sera and their IgGs promoted the macrophage metabolism of CNS and PNS myelin almost equally. The cultured macrophages converted about 3% of untreated CNS myelin and about 6% PNS myelin cholesterol to cholesterol ester. Under phase contrast microscopy it was noted that vesicles of CNS myelin appeared to bind individually to macrophages, whereas PNS myelin vesicles tended to self-associate to form large clumps which were bound to macrophages. Binding studies showed PNS myelin to be bound more firmly to macrophages than CNS myelin. The presence of high amounts of glycoprotein in PNS myelin may account for this difference in binding to macrophages, either through self-adhesion of the PNS myelin particles, or by the presence of carbohydrate receptors in macrophages. These results may define a role for antibody in myelin destruction in cell-mediated demyelinative diseases, and may be relevant to the differences seen in vivo between the rates of demyelination of peripheral and central nerves.

Key words: myelin basic protein, macrophage, galactocerebroside, sciatic nerve

INTRODUCTION

The macrophage is believed to be the ultimate destructive agent of myelin in cell-mediated demyelination. Phagocytosis of myelin by these cells has been observed by electron microscopy in experimental allergic encephalomyelitis (EAE) (Lampert and Carpenter, 1965; Lampert, 1967), in multiple sclerosis (Prineas and Connell, 1978; Raine, 1985), in experimental allergic neuritis (EAN) (Lampert, 1969), and Guillain-Barre syndrome (Prineas, 1981). It has been suggested that antimyelin IgG may serve as a link between the macrophage and the myelin sheath in both multiple sclerosis (Prineas and Graham, 1981) and experimental allergic encephalomyelitis (Epstein et al., 1983). We have previously shown that antibody raised against whole CNS myelin, myelin basic protein (MBP), and galactocerebroside (GC) will greatly enhance the uptake and metabolism of ^{14}C-labeled myelin by macrophages in vitro (Trotter et al., 1986). Not only was antibody-treated myelin phagocytized to a greater extent than untreated myelin or preimmune serum-treated myelin, but conversion of the radioactive lipids of the myelin to radioactive cholesterol ester and triglyceride proceeded much faster than with the control myelin. We found, in the course of these experiments, that antiserum to PNS myelin was as effective as antiserum to CNS myelin in stimulating uptake and metabolism of CNS myelin by macrophages. In studies described here we carry these investigations further by examining the phagocytosis and metabolism of PNS myelin by macrophages in the presence of various specific antisera and IgGs and comparing the results with those from similar studies with CNS myelin.

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METHODS AND MATERIALS

Preparation of Radiolabeled CNS and PNS Myelin

CNS myelin was radiolabeled by intracerebral injection of 75 μCi [1-^{14}C]sodium acetate (5.8 mCi/mm) (New England Nuclear, Albany, NY), into 18 day Wistar rats. After 3–4 days purified myelin was prepared from the brains and spinal cords as previously described (Trotter et al., 1986). For preparation of PNS myelin, sciatic nerves were dissected from 21–23 day old Wistar rats, chopped very fine, and about 75 mg chopped nerve was incubated with shaking in 1.5 ml Krebs Ringer bicarbonate containing 10 mM glucose and 100 μCi [1-^{14}C]sodium acetate. After 3 hr the nerves were centrifuged free of the medium, washed several times in cold deionized water, and purified myelin was prepared on a single sucrose gradient as previously described (Smith, 1980). Purified PNS myelin was also prepared simultaneously from unlabeled adult sciatic nerves and was used to dilute the labeled myelin to a specific activity of 750,000 dpm/mg myelin protein. This myelin after suitable dilution was pelleted in 2 mg aliquots and stored at −70°C until ready for use.

Preparation of Antisera

Antisera to whole CNS and PNS myelin were raised by immunization of white New Zealand rabbits with 1.5 mg purified CNS or PNS myelin in Freund's complete adjuvant. Subsequent immunizations alternated similar dosages in complete or incomplete adjuvant at 6 week intervals with bleeding 10 days after immunization up to a year, during which time no signs of EAE or EAN were observed. The antisera stained most of the major myelin proteins on Western blots (Towbin et al., 1979). Antiserum to MBP and P_2 protein were raised by a similar method using porcine myelin basic protein (generous gift of Lawrence F. Eng) or P_2 purified from beef spinal root myelin (Curtis et al., 1979) as the antigen. The basic protein antiserum stained the four myelin basic proteins of PNS myelin separated by electrophoresis and blotted to nitrocellulose, while antiserum to P_2 stained only a single band running slightly in front of the small myelin basic protein on the blot. Antisera to galactocerebroside (GC) was raised according to the method described by Benjamins et al. (1987). The serum immunostained GC spotted on nitrocellulose paper, but did not stain glucocerebroside or sphingomyelin. IgG was prepared from the various sera using a protein A affinity column (Bio-Rad, Richmond, CA). The antibody to P_0 has been described previously (Smith and Perret, 1986).

Macrophage Cultures

Thioglycollate-elicited macrophages were obtained from adult Wistar rats, adhered to plastic tissue culture plates, and cultured in N2 medium (Bottenstein and Sato, 1979) as previously described (Trotter et al., 1986). Aliquots of myelin (0.125 mg protein) were preincubated with the various sera or IgGs and added to each culture plate. At 8, 16, 24, and 30 hr the cultures were scraped off with the culture medium using several ice cold deionized water washes, and the total mixture was lyophilized. For the binding studies the medium was removed from the cultures and saved, and the cultures washed twice with room temperature phosphate-buffered saline. These washes were combined with the medium and the total adjusted to 10 ml. The cells were removed from the plate with two 1 ml portions of 1% sodium dodecyl sulfate. The cells and aliquots of the supernatant were then counted for radioactivity.

Lipid Separation

The lyophilized mixtures were extracted with chloroform–methanol (2:1) and washed according to Folch-Pi et al. (1957); the lipid mixtures were taken to dryness under a stream of nitrogen, dissolved in a small amounts of chloroform methanol (2:1), and the entire mixture was separated by thin layer chromatography (TLC) with petroleum ether–diethyl ether–acetic acid (80:20:1) as the solvent phase. The separated lipids were visualized with iodine vapor, and with the use of appropriate standards, the spots corresponding to cholesterol ester, triglyceride, free fatty acid, cholesterol, and phospholipids + galactolipids which remained at the origin were scraped into scintillation vials, and the radioactivity was determined in a scintillation counter as described by Trotter et al. (1986). Correction for quench was calculated by the channels ratio procedure. We have previously determined in CNS myelin studies that one-third of the dpm in cholesterol ester was contributed by fatty acid, while two-thirds of the radioactivity was in the cholesterol moeity (Trotter et al., 1986). We ascertained that splitting of the cholesterol ester derived from PNS myelin with cholesterol esterase and separation of the radioactive cholesterol and fatty acid by TLC showed an identical proportional division of the radioactivity. Therefore the fraction of cholesterol esterified from both CNS and PNS myelin was approximated by calculating two-thirds dpm cholesterol ester/(dpm cholesterol + two-thirds dpm cholesterol ester). Levels of significance were determined by Student's t test.

RESULTS

Effect of Antisera

Peripheral nerve myelin pretreated with antiserum to PNS myelin was phagocytized and metabolized by macrophages to produce increasing amounts of cholesterol ester and triglyceride during the course of 30 hr

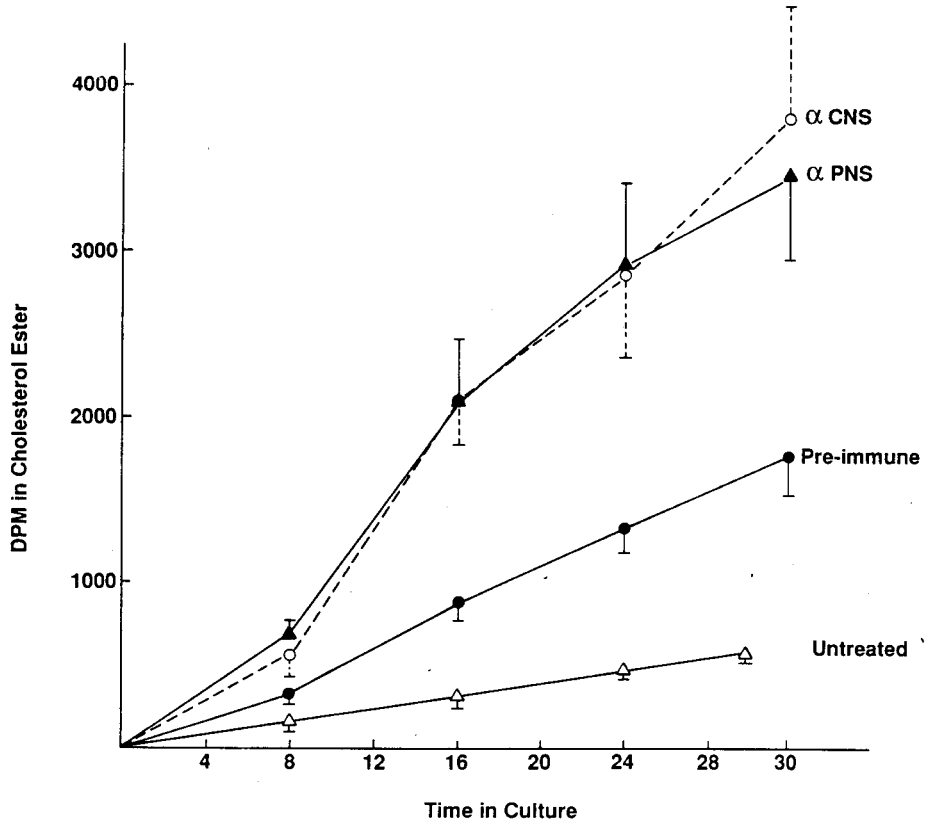

Fig. 1. Time course of production of cholesterol ester by cultured macrophages incubated with PNS myelin opsonized with antiserum to PNS or CNS myelin or preimmune serum, compared with that from untreated myelin. Each point represents the average ± SEM of three separate experiments.

incubation (Figs. 1 and 2). Antiserum to CNS myelin stimulated PNS myelin lipid metabolism equally well, while pretreatment with preimmune serum was much less effective. From untreated myelin the macrophages produced about 15% as much cholesterol ester, and about 30% as much triglyceride as that pretreated with anti-myelin antibody. These results complement our earlier findings that anti-CNS and anti-PNS myelin antibody stimulated equally the production of these neutral lipids from CNS myelin (Trotter et al., 1986).

A number of specific antibodies to PNS myelin constituents were tested to determine their opsonizing effect on myelin compared to the antibody to whole PNS myelin. Two antisera, those to GC and to MBP, were especially effective in promoting production of cholesterol ester and triglyceride by macrophages (Fig. 3). GC antiserum was as effective as that to whole PNS myelin, while MBP antiserum was only slightly less active. All four antisera promoted the cholesterol ester production to an extent significantly higher than the preimmune antiserum ($P < 0.02$ or less). Antiserum to P_0, on the other hand, was about as effective as preimmune serum in promoting neutral lipid production, to about 2.5- to 3-fold that from untreated myelin in this series. P_2 antiserum had some stimulative effect, but this was highly variable, and did not differ significantly from that of the preimmune serum (Fig. 3).

Active factor in serum

To determine the active opsonizing component, the antisera were pretreated in several ways. A thorough dialysis of the antisera before preincubating with myelin did not alter the activity, nor did heat treatment at 56°C for 30 min diminish the stimulatory effect, except for the preimmune serum which occasionally lost some activity (data not shown). Complement, therefore, seems not likely to be an important opsonizing factor in the antibody treatment, but may have some effect in nonspecific opsonization as with the preimmune serum.

In most cases IgG prepared from the individual antisera contained most of the activity contained in antiserum to PNS myelin, CNS myelin, MBP, and GC when cholesterol ester production from either CNS and PNS myelin was compared (Fig. 4). Some exceptions included the IgG from antiserum to GC for PNS myelin, the anti-PNS IgG for CNS myelin, and the preimmune

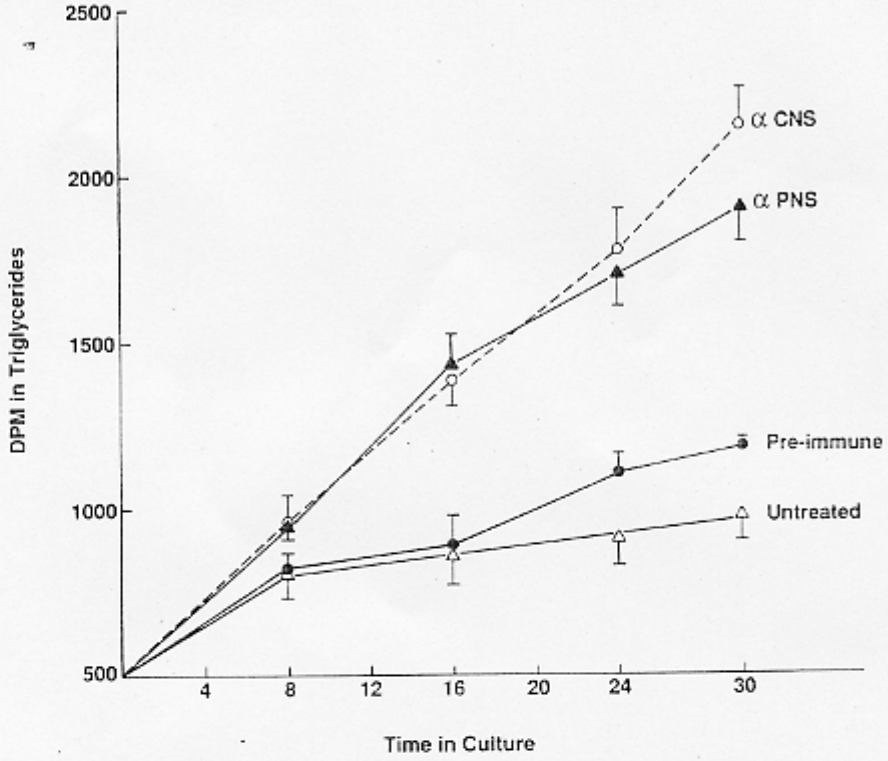

Fig. 2. Production of triglyceride by macrophage cultures as in Figure 1.

IgG for both PNS and CNS myelin, all of which exerted significantly less opsonizing effect than their corresponding serum. The differences between the effects of the sera and IgG should not have been due to loss of complement, since heating the sera did not appear to affect the activities of the antisera. ELISA assays of the anti-GC and anti-PNS sera did not show the presence of IgM. It was concluded that the lesser effects may have been due to the vulnerability of the antibody to losses during IgG isolation, although the decreased effects of the IgGs for GC and PNS myelin were not seen in both CNS and PNS myelin. As might be expected, P_2 antiserum had a larger effect on PNS myelin than on CNS myelin, although the average effect on PNS myelin was only slightly higher than with preimmune serum. P_2 IgG was not prepared. Rat PNS myelin contains only small amounts of P_2, which may account for the slight effect.

The values in Figure 4 are expressed as percent of cholesterol esterified in order to make valid comparisons between the antibody effects on CNS and PNS myelin which differed in their specific radioactivities. A maximum of 30% of the cholesterol contained in the myelin was converted to cholesterol ester at the end of 30 hr. In a few experiments where the incubation time was extended to 48 hr the amount of [^{14}C]cholesterol ester continued to increase linearly (data not shown). The amount of myelin present, therefore, was not limiting throughout the course of the incubation.

Although the cholesterol ester production from untreated myelin was relatively low, it was apparent that twice as much was produced from PNS as from CNS myelin. When the time course of cholesterol production was compared, it was determined that after some lag, the rate of cholesterol ester production from PNS myelin increased to reach a level of over 6% of total cholesterol esterified, while half as much was produced from CNS myelin (Fig. 5). From observation of the cultures it was noted that the administered PNS myelin was bound to the macrophages differently than the CNS myelin. Whereas individual vesicles of CNS myelin appeared to bind independently to the macrophages, thus resembling bunches of grapes, the PNS myelin seemed to self-associate in large bodies which were bound to the macrophages (Fig. 6A and B). The amount of cell-associated radioactivity in cultures containing either of the two kinds of untreated myelin was measured at the end of 8 and 30 hr. The uptake was greatest in the first 8 hr where over 30% of the radioactivity in PNS myelin became cell-associated compared to 8% of that of the CNS myelin. These amounts increased up to 30 hr, but at a slower rate (Fig. 7). When twice as much radioactive myelin was incubated with the macrophages, the same proportion of PNS myelin became cell-associated at 8 hr, whereas that of CNS myelin was less, indicating a saturation of the uptake mechanism for CNS myelin, but not for PNS myelin. At 30 hr the proportion of cell-associated PNS

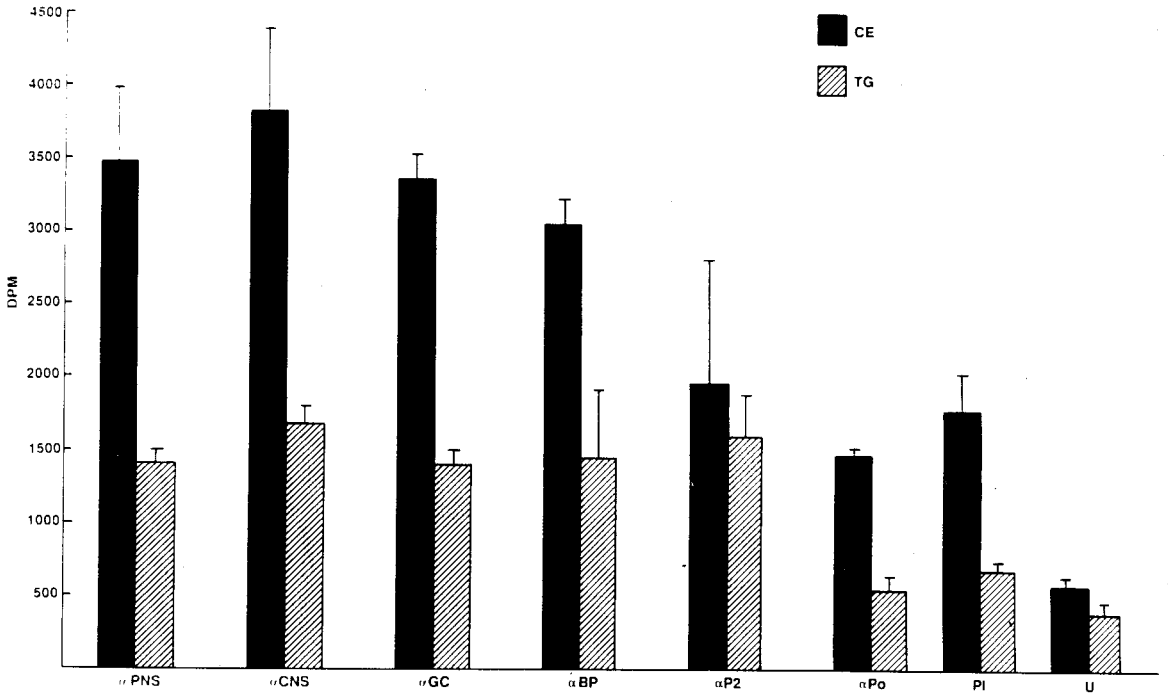

Fig. 3. Production of cholesterol ester (CE) and triglyceride (TG) by cultured macrophages after a 30 hr incubation with myelin opsonized with various antisera. α GC, galactocerebroside antiserum; α BP, myelin basic protein antiserum; α P_2 and P_0 are antisera to the designated myelin proteins; PI, preimmune; U, untreated myelin. Each value represents the average of results of 2–6 different experiments ± SEM. Cholesterol ester levels for antisera to GC, BP, CNS, and PNS are significantly higher than that for PI or untreated ($P < 0.02$ or lower).

myelin was less, but not half, therefore addition of higher amounts of myelin promoted more total uptake. The uptake of CNS myelin, however, appeared to be saturated by the lower myelin levels.

DISCUSSION

We have shown previously that antiserum to whole CNS myelin, whole PNS myelin, and antibodies to GC and MBP stimulate the uptake and phagocytosis of CNS myelin, the esterification of myelin cholesterol, and formation of triglyceride from myelin lipids by cultured macrophages to a much greater extent than does normal serum (Trotter et al., 1986). The experiments described here show that opsonization of both CNS myelin and PNS myelin with these same antibodies promotes increased phagocytosis and metabolism equally effectively with the two kinds of myelin. Strangely, the antibodies to the main structural proteins of the two myelins, proteolipid protein (CNS) and P_0 (PNS) are without effect, as was the antibody to the myelin-associated glycoprotein. It can be calculated that in the course of 30 hr each macrophage culture (about 2×10^6 cells) can convert as much as 37.5 μg of cholesterol to cholesterol ester (125 μg myelin protein/plate = 500 μg myelin lipids containing about 25% cholesterol). Higher levels were obtained with increased incubation times. Although the amounts of triglyceride produced did not show such striking changes as the cholesterol ester, the trends were approximately the same.

Opsonins, which promote the formation of an adhesive bond between the particle to be ingested and the phagocytic cell, are of two types in serum, one of which is heat-labile, the other, heat-stable. It is generally believed that heat-stable opsonins are immunoglobulins, while the heat-labile factors are due to complement components (Absalom, 1986). In the experiments described here the opsonic activity of serum which promotes myelin phagocytosis by macrophages could be equalled, in most cases, by substituting IgG derived from that serum. Heating the serum did not diminish the activity, therefore complement is probably not the principal opsonizing factor in this system, thus the promotion of both PNS and CNS myelin phagocytosis and metabolism can be mainly ascribed to IgG. In the absence of serum, however, another factor clearly appears to be present in PNS myelin which promotes association with the macrophages. As much as 50% of the PNS myelin vesicles became cell-associated, whereas only 20% of the CNS vesicles associated with the macrophages in the same 30 hr period.

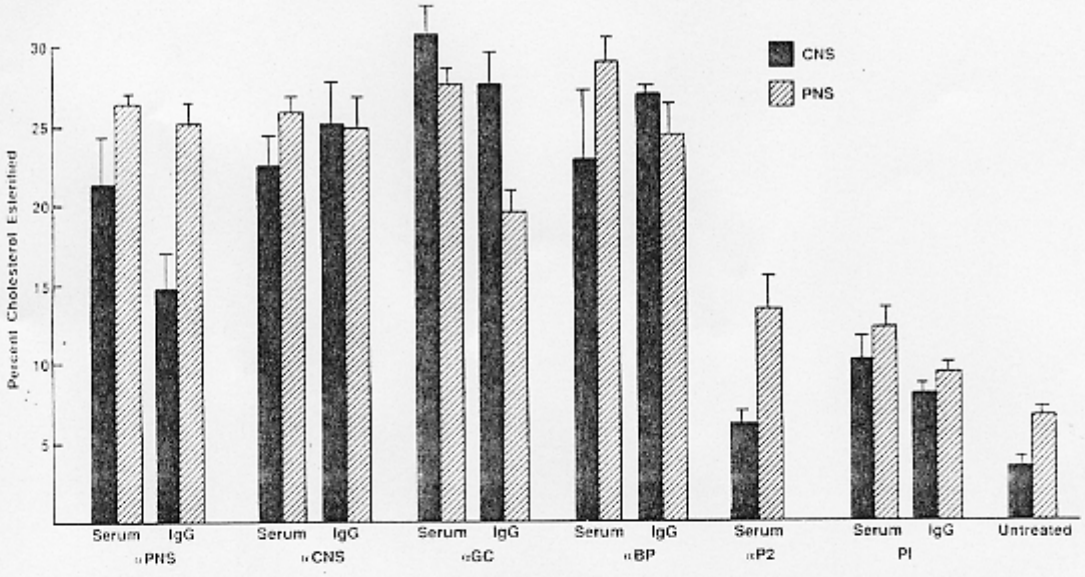

Fig. 4. Comparison of percent of cholesterol esterified by macrophage cultures incubated for 30 hr with CNS or PNS myelin opsonized with antisera or their derivative IgGs to various myelin constituents. Each value represents the average of 2–6 separate experiments. Cholesterol ester values for antisera and IgGs for PNS, CNS, GC, and BP are significantly higher than the corresponding value for PI ($P < 0.05$ or lower).

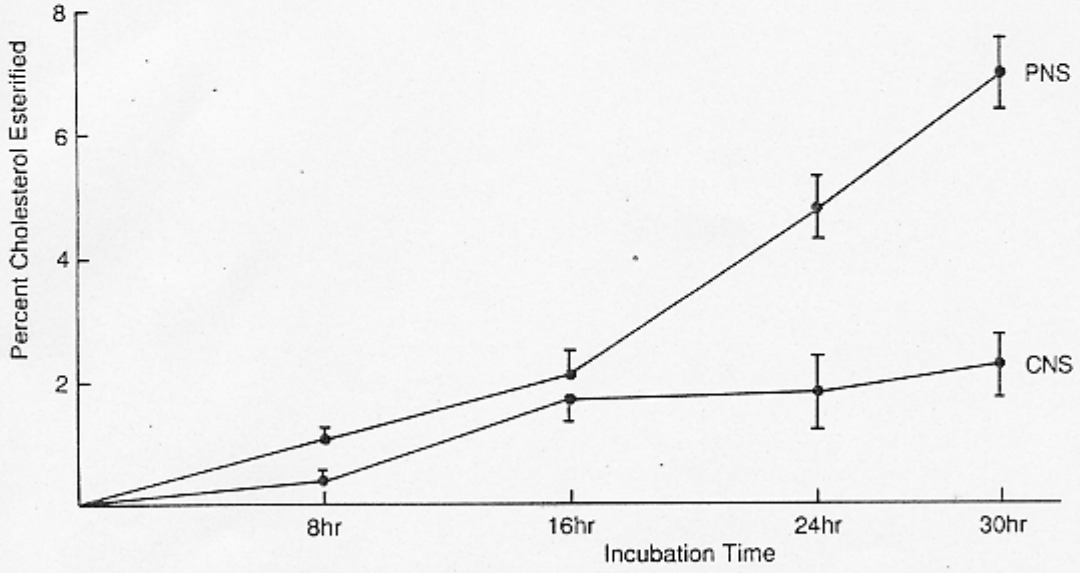

Fig. 5. Thirty hour time course of production of cholesterol ester from untreated PNS and CNS myelin incubated with macrophages.

The main difference between PNS and CNS myelin is the high content of glycoprotein in PNS myelin (60–70% of the total protein), whereas CNS myelin contains much less glycoprotein (about 5%). One factor possibly contributing to the adherence of myelin to macrophages is the tendency of PNS myelin vesicles to aggregate as observed shortly after addition to the cell culture. Thus, when one or a few myelin vesicles bind to the macrophage, many more vesicles may also adhere to the bound myelin. Sone evidence for this mechanism can be discerned by inspection of Figure 6B. D'Urso et al. (1990) and Filbin et al. (1990) have recently described a homophilic adhesion of mammalian cells transfected with P_0 cDNA. This adhesion appeared to be due to P_0 ex-

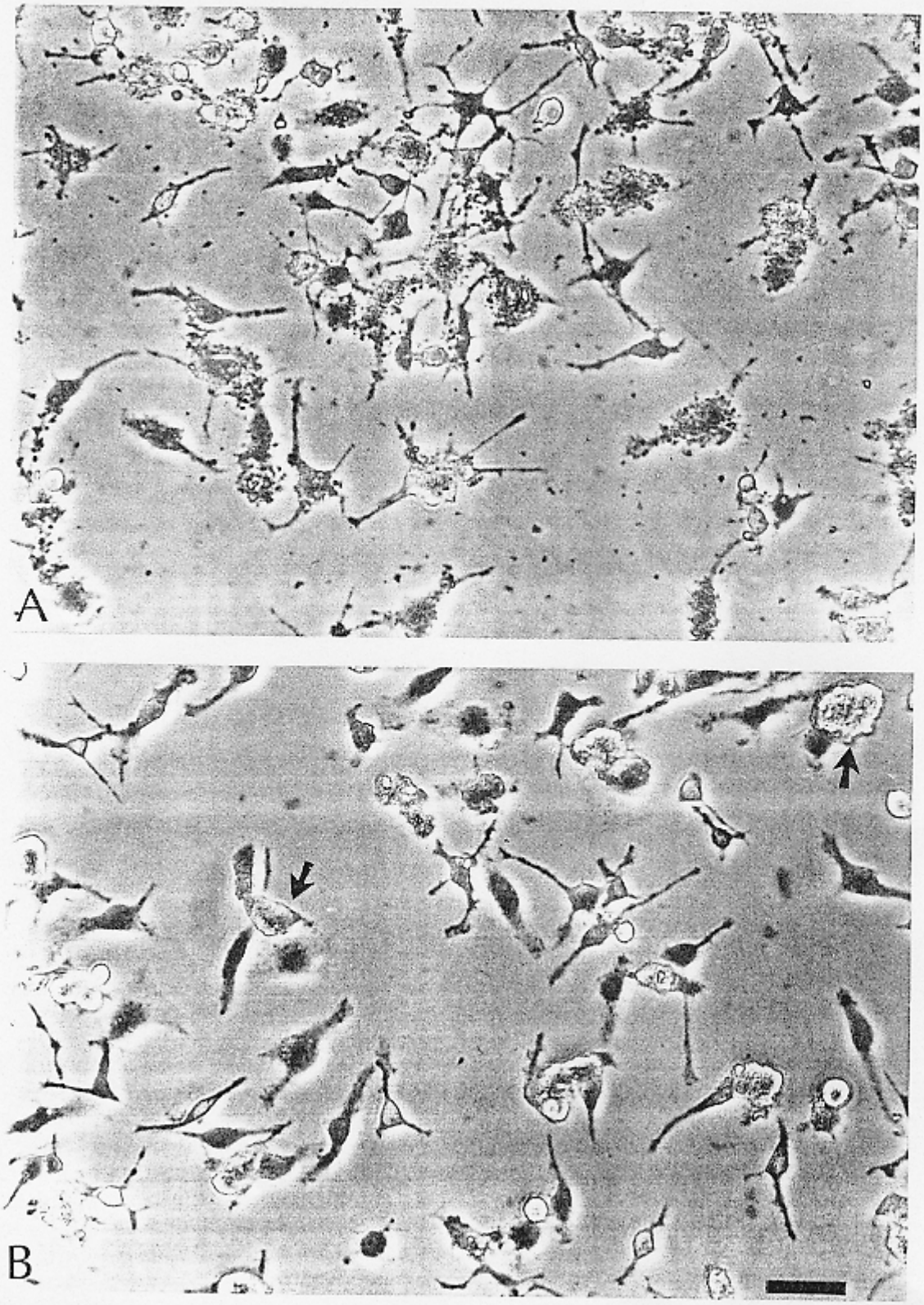

Fig. 6. Rat peritoneal macrophages after a 30 hr incubation with CNS (**A**) or PNS (**B**) myelin. (**A**) Individual CNS myelin vesicles are attached independently to the exterior of macrophages resembling bunches of grapes. Some myelin vesicles are still unattached. (**B**) PNS myelin vesicles appear to self-associate into larger bodies (arrows) which are attached to the macrophage exterior. Very few freely floating vesicles are present. Bar = 40 μm.

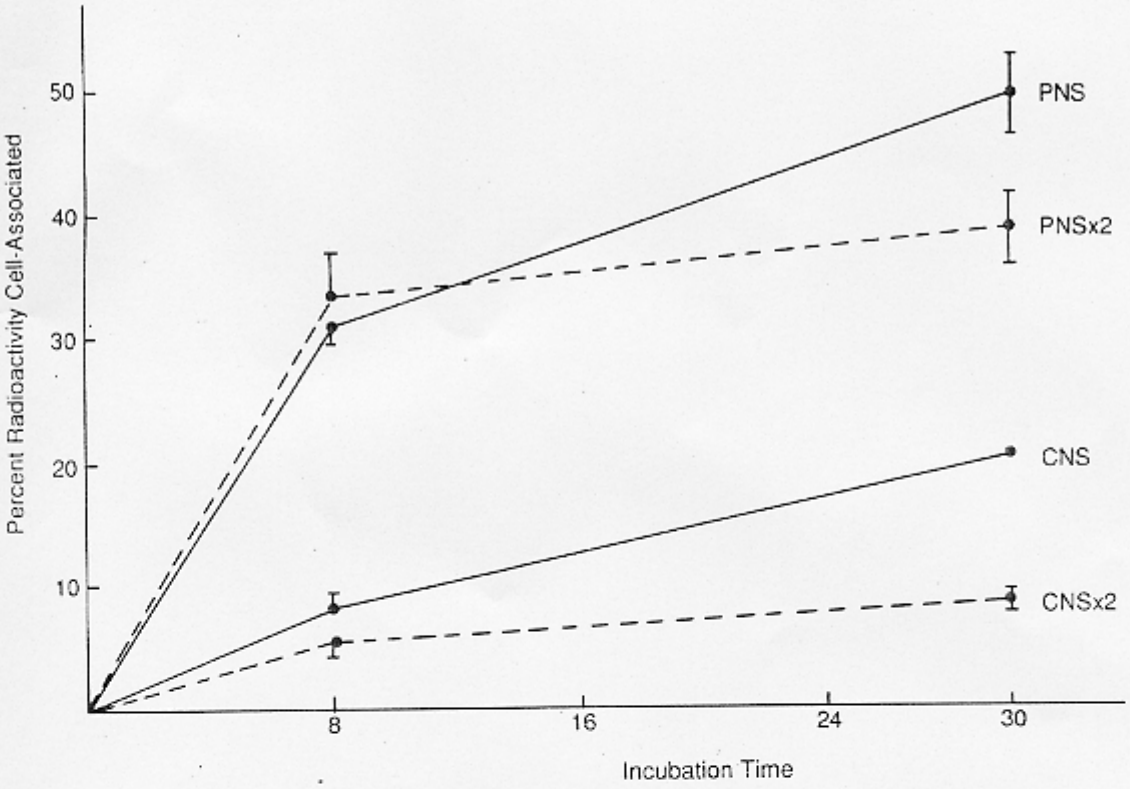

Fig. 7. Time course of cell association of CNS and PNS myelin with macrophages as measured by the proportion of radioactivity firmly bound after directing a stream of room temperature PBS onto the cultures.

pressed at the cell membrane and was highly concentrated at cell–cell interfaces (D'Urso et al., 1990). Thus this adhesive property of P_0 may contribute to an increase in PNS myelin–macrophage association. This effect may be an artifact of the method of myelin presentation in these experiments. The augmented uptake of PNS myelin could also be attributed to carbohydrate receptors which are known to be present on macrophages. A number of these lectin-like receptors show specificities for different sugars, and serve mainly an endocytic function (reviewed by Gordon et al., 1988).

Although PNS myelin was effectively taken up, the extent of actual internalization could not be determined from Fig. 6A and B. Although much of the PNS myelin was cell associated, relatively little of the PNS myelin cholesterol was esterified (about 6%), compared to that of myelin phagocytosed by IgG opsonization (about 30%) which is known to involve Fc receptors. Phagocytosis of myelin by macrophages in experimental allergic encephalomyelitis has been shown to involve the attachment of myelin to coated pits (Epstein et al., 1983). The more extensive binding of PNS myelin compared to CNS myelin may be accounted for by carbohydrate receptors. Whether these can function in PNS myelin internalization in the absence of antiserum is under further investigation.

Others have observed that macrophages are far more active in injured peripheral nerves than in central nerves. Thus, Perry et al. (1987) observed rapid recruitment of macrophages and phagocytosis of myelin after crush injury to the sciatic nerve, while a similar injury to the optic nerve attracted few macrophages. In the latter case myelin removal was very slow. There is some evidence that IgG may not be involved in Wallerian degeneration in peripheral nerves. In experiments described by Hann et al. (1988) antibody production was suppressed by antibodies against IgM in mice, and by bursectomy in chicks, and myelin degeneration was observed during Wallerian degeneration in the two species. There was little difference between the rates of myelin phagocytosis by monocytes in the treated and untreated animals. It is more likely that immunoglobulin may be involved in pathologies of peripheral nerves involving immune responses. Thus cultured macrophages preincubated with serum from rabbits with experimental allergic neuritis rapidly phagocytized purified bovine or rabbit PNS myelin, while those incubated with normal serum or serum from adjuvant-injected animals could not (Sobue et al.,

1982). We have also found in our system that EAN serum from Lewis rats opsonizes PNS myelin, leading to rapid uptake and synthesis of large amounts of cholesterol ester (unpublished results). Whether such mechanisms may be involved in other kinds of peripheral neuropathies involving immunoglobulins is at present unknown.

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