This is a particularly powerful approach to obtaining the complete primary sequence of proteins such as PDGF that are available in such small quantities that obtaining the complete sequence from the protein itself is difficult at best. The utility of the method is illustrated by the recent success in cloning the complementary DNA's of the acetylcholine receptor subunits with the use of synthetic DNA probes based on amino-terminal sequence data (*18*). It can also be used to synthesize polypeptides (*19*) for the generation of antibodies to PDGF that might be useful in functional studies, cloning experiments, and radioimmunoassay of PDGF.

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Cytotoxicity of a Perfluorocarbon Blood Substitute to Macrophages in vitro

Abstract. *Murine macrophage and macrophage-depleted splenocyte cultures were incubated under ambient oxygen with a commercially available perfluorocarbon blood substitute. The perfluorocarbon preparation was found to be selectively cytotoxic to macrophages. This finding may be significant in view of the preliminary therapeutic usage of these preparations. In addition, perfluorocarbons may be useful as a means of selectively removing macrophages from tissue and organ cultures.*

Liquid perfluorocarbons have the interesting property of dissolving a far greater volume of oxygen than other liquids at comparable partial pressures. Whereas normal saline or blood plasma dissolves about 3 percent oxygen (based on the volume ratio of gas to liquid) and whole blood about 20 percent, pure perfluorocarbon can dissolve 40 percent or more (*1*). This phenomenon has led to the development of stable, aqueous emulsions of these chemicals for use as oxygen transport fluids. They have been tested in experimental animals as breathing liquids and as substitutes for red blood cells (*2, 3*). Recently, perfluorocarbon emulsions have been used therapeutically as blood supplements in patients who required emergency surgical treatment or who refused blood transfusions (*4*). These initial clinical studies have shown little toxicity with the exception of a report of pulmonary crisis believed to be due to complement activation (*5*).

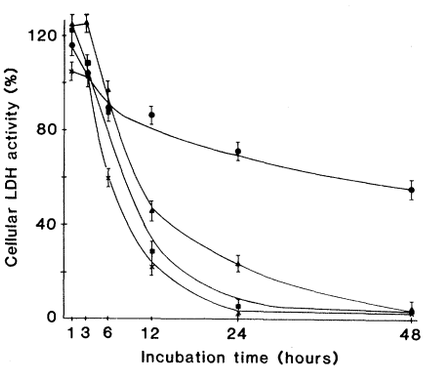

Fig. 1. Percentage decrease in cellular LDH activity of macrophage cultures incubated with (●) 5 percent, (▲) 10 percent, (■) 15 percent, and (x) 20 percent FC-43. For each assay, 1×10^6 cells in 1 ml were cultured in a 2.0-cm^2 multiwell tissue culture plate. At the end of the incubation the cells were washed, lysed, and assayed for LDH by measuring the reduction of pyruvate to lactate, using reduced nicotinamide-adenine dinucleotide (NADH) as the coenzyme (*9*). Activity was calculated from the rate of the change in absorbance at 340 nm as measured in a Beckman Acta III spectrophotometer. The percent activity is expressed in terms of the ratio of LDH activity of the fluorocarbon-incubated culture over a control culture containing no fluorocarbon for that time point. Error bars denote the standard error of the mean for two assay determinations.

Reasoning that the particulate nature of perfluorocarbon emulsion might lead to its uptake by the reticuloendothelial system, we were concerned about adverse effects that perfluorocarbon therapy might have on macrophage function. Animal studies have shown that a large percentage of the administered material is retained in the liver and the spleen with an elimination half-life of several weeks (*6*).

We studied the effect on murine leukocytes in vitro of a commercially available preparation of artificial blood containing as principal ingredients FC-43 (perfluorotributylamine, 25 percent, weight/vol-

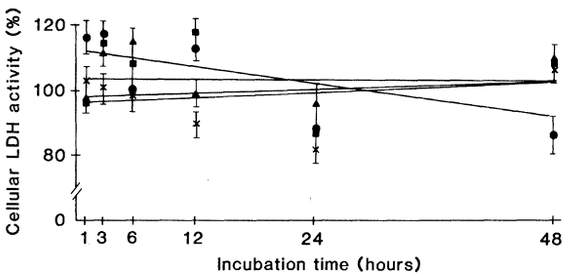

Fig. 2. Percentage decrease of cellular LDH activity for mouse splenocytes depleted of adherent cells and cultured with (●) 5 percent, (▲) 10 percent, (■) 15 percent, and (x) 20 percent FC-43. For each assay, 2×10^6 cells in 1 ml were cultured in a 2.0-cm^2 multiwell plate. Error bars denote the standard error of the mean for two assay determinations.

ume) emulsified with Pluronic F-68 (polyoxypropylene-polyoxyethylene copolymer, 3.2 percent, weight/volume) (*7*).

Macrophages were obtained from peritoneal exudates of mice of the NCS strain (Rockefeller University colony) (*8*). After removal of nonadherent cells, cells were cultured overnight at a density of 1×10^6 per milliliter in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 percent heat-inactivated fetal bovine serum and 10 m*M* Hepes. The same medium supplemented with increasing percentages of FC-43 was then added and incubation continued for 48 hours in a humidified incubator containing ambient air and 5 percent carbon dioxide. Morphologically, an increase in cytoplasmic vacuolation was observed in the cultures, followed by a loss of cellular adherence to the culture surface and cytolysis.

The cytotoxic effect was quantitated by assaying lactate dehydrogenase (LDH) in the remaining cells. After increasing incubation times and in the presence of different percentages of FC-43, the adherent cells were washed free of medium and of cellular debris. The cells were then lysed by the addition of 0.2 ml of distilled water and frozen at −80°C until assay.

The effect of increasing percentage of FC-43 and increasing incubation time on macrophage integrity is illustrated in Fig. 1. Similar activity curves were obtained with macrophages separated from the supplemented medium by centrifugation, and a corresponding increase in LDH activity in the medium was observed. This indicates that the loss of cellular LDH activity is due primarily to cytolysis and not simply to a loss of cellular adherence. Figure 1 shows that the LDH activity of the treated cells is higher than that of the controls for up to 3 hours but begins to fall as cells lose adherence and die. By 24 hours, the 15 and 20 percent perfluorocarbon cultures are almost devoid of cells, while the toxicity of the 5 percent perfluorocarbon culture has begun to level off. In this case, either a more susceptible subpopulation of cells has been killed or the remaining cells have adapted to the presence of this level of perfluorocarbon. Since the rate of cell death increases with increasing perfluorocarbon concentration, the cytotoxic mechanism must be dose-dependent.

Figure 2 represents an identical experiment performed with murine lymphocytes depleted of adherent cells. Splenocytes were obtained from NCS mice (*10*) and depleted of adherent macrophages by two successive 3-hour incubations on plastic petri dishes. After overnight incubation in DMEM containing 10 percent fetal bovine serum and 10 m*M* Hepes, cells were centrifuged and fresh medium containing different percentages of FC-43 was added. After incubation, lymphocytes were washed, lysed, assayed for LDH, and the activity compared to that of control cultures containing no FC-43. No cytotoxic effect was seen under the conditions observed to be lethal for macrophages.

Another experiment was done to investigate the nature of the perfluorocarbon toxicity to macrophages. This is illustrated in Fig. 3. Macrophage cultures were incubated for 6 hours with 10, 15, and 20 percent perfluorocarbon, after which the cells were washed, the emulsion replaced with fresh medium, and the cells assayed for viability after further incubation. Cells in the 20 percent culture continued to die at the same rate as in Fig. 1. Cell death in the 10 and 15 percent cultures slowed, however, and leveled off after 18 hours. It seems most likely that the surviving cells either had not accumulated irreversible toxic damage or represented a subpopulation of cells more resistant to the perfluorocarbon.

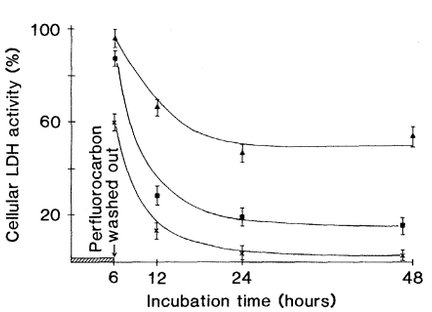

Fig. 3. Percentage decrease of macrophage LDH activity as a function of time after a 6-hour exposure to (▲) 10 percent, (■) 15 percent, and (x) 20 percent FC-43. Error bars denote the standard error of the mean for two assay determinations.

An interesting question is the mechanism of the cytotoxic action of perfluorocarbon. The average particle diameter of the FC-43 preparation is 0.1 μm (*1*), which would allow the particles to be taken up by either phagocytosis or pinocytosis (*11*). Intracellularly, it is unlikely that the perfluorocarbon is chemically toxic, as the high stability of the C–F bonds (energy content, 116 kcal/mole) would make them virtually unreactive (*12*). Alternatively, the perfluorocarbon may interact with phospholipid components and disrupt the phagolysosomal membrane, leading to cell destruction.

Another possibility is that the cytotoxic effects are due to free radical mediators generated by oxygen toxicity. Perfluorocarbon particles may carry high levels of dissolved oxygen directly into the macrophage. Although macrophages have enzymatic mechanisms for detoxifying oxygen-generated free radicals (*13*), the ingested perfluorocarbon may produce high intracellular oxygen gradients that overwhelm these defenses. Nonphagocytic cells would be spared this damage. We observed that incubation of macrophages with 100 percent oxygen at a low level of perfluorocarbon (5 percent) enhanced cytotoxicity. Control cultures containing perfluorocarbon but incubated under nitrogen showed greater survival (*14*).

In conclusion, we observed that the perfluorocarbon preparation FC-43 was cytotoxic to murine macrophages incubated under ambient oxygen conditions. Although the viability of lymphocytes was not decreased, functional studies should be done to ensure that nonphagocytic cells are not affected by this material. The perfluorocarbon levels we used were less than or comparable to those used in recent clinical studies (*4*). It would be important to determine to what degree macrophage function is compromised in vivo in these patients.

In experimental work, perfluorocarbon may prove useful as a means of selectively removing phagocytic cells from cultures. For example, the successful removal of macrophages bearing I-region associated (Ia) antigens is believed to be one mechanism for enhancing allograft survival after transplantation (*15*). Culture of thyroid or islet of Langerhans allografts prolongs graft survival, presumably because oxygen selec-

tively destroys Ia-bearing passenger lymphoid cells (*16*). Incubation or perfusion of graft tissue with perfluorocarbon may be a means of efficiently removing oxygen-sensitive lymphoid cells before transplantation.

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Adenosine Receptors: Autoradiographic Evidence for Their Location on Axon Terminals of Excitatory Neurons

Abstract. *Adenosine receptors were made visible on light microscopy by autoradiography with tritiated cyclohexyladenosine. In the cerebellum, adenosine receptors were absent in Weaver mice, which lack granule cells, and were displaced in Reeler mice, which have displacements of granule cells. Thus, adenosine receptors appear to be located on the axon terminals of excitatory granule cells in the cerebellum. Removal of one eye of a rat depleted adenosine receptors in the contralateral superior colliculus, suggesting that the receptors occur on axon terminals of excitatory projections from retinal ganglion cells. The presence of adenosine receptors on excitatory axon terminals may explain synaptic inhibition by adenosine and the behavioral effects of xanthines.*

As a neuromodulator, adenosine inhibits the release of excitatory transmitter and also has postsynaptic effects (*1*). Behavioral stimulation by xanthines such as caffeine is linked to the blockade of adenosine receptors (*1, 2*). Several ^{3}H-labeled ligands have been used to identify these receptors (*3*). We located adenosine receptors in vitro by autoradiographic analysis (*4*) using ^{3}H-labeled cyclohexyladenosine ([^{3}H]CHA) (*5*); a similar finding was reported in a preliminary communication by others (*6*). In the present study using neurologic mutant mice as well as selective brain lesions in rats, we provide evidence for an association of adenosine receptors with cerebellar granule cells and retinal ganglion cells.

The cerebellum contains five types of neurons. Four of these (stellate, Golgi, basket, and Purkinje) are inhibitory and appear to use γ-aminobutyric acid as their neurotransmitter (*7*). The fifth type, the granule cell, is the only excitatory neuron in the cerebellum, but is the most frequently occurring cell type, accounting for about 80 percent of cerebellar neurons. The excitatory neurotransmitter of granule cells appears to be glutamic acid (*8*).

In normal rat cerebellum, the highest concentration of adenosine receptors was found in the molecular layer, with a lower concentration in the granule cell layer (*5*), observations we now confirm in mouse cerebellum. These receptors might be located on any of the distinct neuronal types.

To determine the location of cerebellar adenosine receptors, we used mutant mice with specific autosomally recessive neuronal defects. "Nervous" mice show a 90 percent loss of Purkinje cells, with other cerebellar cell types essentially normal (*9*). The overall pattern and density of [^{3}H]CHA grains in the cerebellum of nervous mice are the same as in littermate controls (data not shown). Thus, adenosine receptors in the cerebellum are not associated with Purkinje cells.

Weaver mice have an 80 percent deficiency of granule cells, with other cerebellar cell types being essentially normal (*9*). There were 70 to 80 percent fewer [^{3}H]CHA grains in the cerebellums of Weaver mice than in littermate controls (Fig. 1), as shown by grain counts.

The loss of adenosine receptors in Weaver mice indicates that the receptors are associated with granule cells. The extremely large number of adenosine receptors in the molecular layer of the normal cerebellum are thus confined to axons and terminals of parallel fibers of the granule cells in the molecular layer. We also examined Reeler mice in which the normal migration of granule cells does not occur so that granule cells and their axons remain in the external granular layer. In Reeler mice, [^{3}H]CHA grains were restricted to the external granular layer, the location of the granule cells (data not shown). This finding confirms an association of adenosine receptors with the axons and terminals of granule cells.

Ganglion cells of the retina are major excitatory neurons that project both to the lateral geniculate body and the superior colliculus. Their transmitter is not clearly established but appears to be one of the excitatory amino acids (*10*). Both the superior colliculus and the lateral geniculate have high densities of [^{3}H]CHA-labeled adenosine receptors (*5*).

To determine whether adenosine receptors might be associated with projections of retinal ganglion cells, we removed eyes unilaterally from Sprague-Dawley rats and conducted autoradiographic analysis of [^{3}H]CHA binding 21 days later (Fig. 2). Enucleation abolished [^{3}H]CHA labeling in the superior colliculus opposite the enucleation. The labeling in the lateral geniculate appeared to be normal on both sides after removal of the eye. Thus, adenosine receptors seem to be associated with excitatory projections from retinal ganglion cells to the superior colliculus. Although transynaptic effects cannot be ruled out, the similar loss of grains observed 4 and 21 days after enucleation fits a presynaptic location of the receptors.

Most ganglion cells are bifurcated, sending some processes to the superior colliculus and others to the lateral geniculate. If adenosine receptors are synthesized by these neurons, receptors would be lost from the lateral geniculate as well as from the superior colliculus. Adeno-