DNA sequence involved in the permutations of the L and S regions of the normal HSV genome. Because we can now make reasonable quantities of these specific HSV fragments quickly, economically, and without working with the infectious virus, structural analyses become much less tedious and complex. Furthermore, the separated DNA strands of cloned fragments for transcription analyses provide a tool not available for HSV before. The identification of proteins by the hybrid arrest method (*16*) with the use of in vitro translation of HSV-1 messenger RNA is simplified with these highly purified DNA fragments. Finally, we expect the new NIH Guidelines for Recombinant DNA Research (*17*) will lower the level of containment for these λ-HSV1 experiments so that these and similar hybrids will be available to most laboratories for further study.

Note added in proof: We have now cloned and identified two new Eco R1 fragments, H and G. In addition, we have cloned in the plasmid pBR322, the 3.4-kpb Bam HI fragment carrying the entire thymidine kinase gene.

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10. The λ-HSV1 hybrids were made by ligation of purified vector arms with Eco R1-cleaved HSV-1 DNA (*11*, *18*). The DNA mixtures were packaged in vitro (*11*) under P4 conditions. Initial plaques were formed on the *E. coli* K12 strain LE392 (*20*) at 38°C and then stabbed with toothpicks to another set of LE392 lawns for small-scale amplification at 38°C. The stabs were picked as agar plugs to Falcon 2063 tubes containing 1 ml of TMG (*11*) and two drops of chloroform. These primary stocks contained about 10^6 to 10^7 phage. For additional amplification, one drop of the primary stock was added to 0.2 ml of a twofold concentrated suspension of fresh overnight culture of DP50*sup*F (*8*) resuspended in 0.01*M* $MgSO_4$. After 5 minutes for phage adsorption, the mixture was added to 75 ml of LB broth containing thymidine (50 μg/ml) and diaminopimelic acid (100 μg/ml) required for growth of DP50*sup*F. The cultures were shaken vigorously at 38°C for 6 to 12 hours or until lysis occurred. They were then treated with 5 ml of chloroform at 38°C for at least 5 minutes and clarified by centrifugation at 4000 rev/min for 15 minutes. Phage particles were concentrated with polyethylene glycol 6000 (*11*, *18*). The phage precipitate was collected by centrifugation and resuspended in about 2 ml 0.02*M* tris-HCl, *p*H 7.4, 0.01*M* $MgSO_4$. The polyethylene glycol was removed with an equal volume of chloroform, and the mixture was centrifuged (4000 rev/min) for 15 minutes. The aqueous layer was removed and mixed in sterile serum vials with lysis buffer at a final concentration of 0.08*M* EDTA, 0.01*M* tris (*p*H 7.4), 0.1 percent SDS, and Proteinase K (0.2 mg/ml). The vials were tightly capped and heated at 55°C for 2 hours. A portion from each vial was tested in accordance with the Building 550 P4 Operations Manual [see (*7*)] for the presence of bacteria that could grow in LB broth supplemented with thymidine and diaminopimelic acid as well as for phages that could form plaques on a DP50*sup*F lawn. We estimated that about 10^{12} viable phage particles were in each vial prior to treatment. Only those vials with no bacteria or phage after 24 hours at 31°C in the sterility test were allowed to be removed from the P4 facility. Sterile vials were immersed in 0.1 percent Clorox solution for at least 5 minutes to decontaminate the vial prior to removal from P4. Once removed, the sealed vials were rinsed with mercaptoethanol buffer to inactivate residual Clorox and the contents treated with ribonuclease (50 μg/ml) (Worthington, pancreatic) for 60 minutes at 37°C, then sequentially extracted with one volume each of phenol, phenol : chloroform (1:1), and chloroform. Two volumes of ethanol were added to the aqueous phase and the precipitated DNA spooled onto a glass rod. The DNA (50 to 100 μg per each 75 ml of lysate) was dissolved in 20 m*M* tris-HCl, *p*H 7.5, 0.1 m*M* EDTA and frozen at − 20°C.
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20. LE392 is our stock number of strain ED8654, a gift from Dr. W. Brammar. The relevant genotype of this strain is *sup*E *sup*F *hsd*R$^-$M$^+$S$^+$ *met*$^-$ *trp*R *lac*$^-$.
21. We thank Dr. B. J. Graham for the defective DNA used; Dr. J. Seidman for advice and materials for some of the in vitro packaging preparations; Dr. David Tiemeier for advice and encouragement; Dr. Neil Wilke for the Bam HI physical map of HSV-1; and Dr. S. Nagle, T. Bryan, J. Brewer, and H. B. Stull for advice and technical support during our stay at the Frederick Cancer Research Center P4 facility.

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Radioimmunoassay of the Insulin Receptor: A New Probe of Receptor Structure and Function

Abstract. *A sensitive and specific radioimmunoassay for the insulin receptor has been developed employing receptor autoantibodies from the serum of a patient with insulin-resistant diabetes. The assay detects insulin binding sites at concentrations as low as 0.1 nanomolar; distinguishes between receptors originating from human placental membranes, human lymphoblastoid cells, and mouse liver membranes; and measures the receptor independently of its binding function. Down-regulation, or loss of binding after exposure to insulin, is associated with loss of immunoreactive receptor.*

Receptors for hormones, neurotransmitters, and other biologically active substances have been studied directly by measuring the specific binding of radiolabeled ligands (*1*). These studies have yielded information about the nature of ligand-receptor interactions and the role of receptors in physiology and disease. It is likely, however, that ligands interact with only a limited region of the receptor molecule, and techniques capable of probing other regions of receptors could help in elucidating receptor function. Toward this end, we have employed a highly specific antibody to the insulin receptor to establish a sensitive and specific radioimmunoassay for the insulin receptor molecule.

The receptor antibodies used in these experiments occur naturally in the serums of some insulin-resistant diabetics, who were described previously (*2*, *3*). The antibodies inhibit binding to both membrane-integrated and solubilized receptors (*2*-*5*), and specifically and completely immunoprecipitate solubilized insulin receptors (*5*). In addition, ^{125}I-labeled antibody binds to cells in proportion to their number of insulin receptors, and this binding is inhibited by insulin and insulin analogs in proportion to their affinity for the insulin receptor (*6*, *7*). Finally, the antibodies mimic a wide range of insulin effects on insulin-responsive cells in vitro (*8*, *9*). This evidence indicates that the antibodies bind directly to the receptor. It suggested to us that the antibody would be a suitable reagent for immunoassay of the receptor.

Insulin receptors were solubilized in Triton X-100 from human placental membranes as previously described (*10*).

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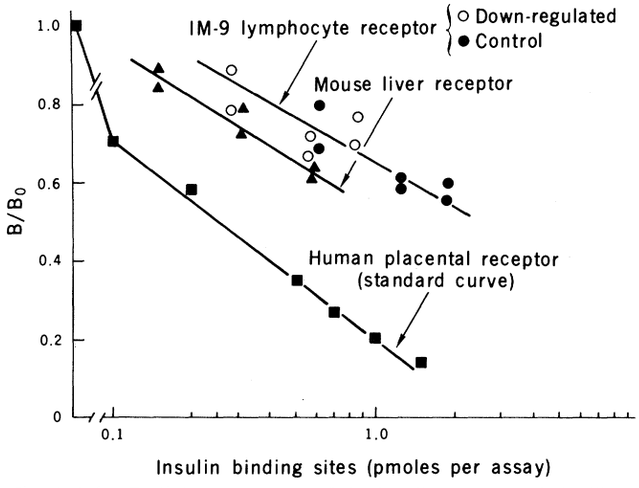
These receptors retain all the properties of the membrane-integrated receptor, including negative cooperativity (*10*). Labeled receptor was prepared by incubating solubilized placental membranes with [^{125}I]insulin followed by gel filtration at 4°C to separate labeled receptor from free insulin. When labeled receptor was incubated with antiserum to receptor, all the receptor was specifically precipitated by subsequent addition of a second antibody to human immunoglobulin (*5*). When this immunoprecipitation is performed in the presence of increasing amounts of unlabeled receptor, the competition of the unlabeled receptor with the labeled receptor for antibody binding can be studied, in accordance with the classical immunoassay scheme (*11*).

Figure 1 shows the effect of increasing concentrations of solubilized insulin binding sites on the immunoprecipitation reaction. In the absence of added unlabeled receptor, 70 to 80 percent of the tracer was precipitated at the dilution of antibody employed. With increasing concentrations of unlabeled placental receptor, the percentage of labeled placental receptor precipitated was decreased. Maximum displacement was 85 percent and was caused by 1.5 pmole of insulin receptor (binding sites); half-maximum displacement was caused by 0.2 pmole of insulin binding sites. The sensitivity was 0.1 pmole of insulin binding sites (~0.1 n*M*), and the interassay variation was less than 10 percent.

Receptors solubilized from mouse liver membranes and from cultured human IM-9 lymphocytes produced displacement curves parallel to the human placental standard curve but shifted to the right by factors of 3 and 7, respectively (Fig. 1), probably because of reduced affinity of the antibody for these receptors. Solubilized cell extracts in which no insulin binding could be detected caused no displacement in the immunoassay (data not shown). Moreover, when solubilized placental membranes were filtered over an insulin-agarose affinity column, the insulin binding activity and the receptor as measured by radioimmunoassay were depleted equally by about 80 percent (Fig. 2). Thus, this appears to be a valid immunoassay for the insulin receptor. Three preparations containing insulin receptors caused parallel displacement in proportion to their content of insulin binding sites, and affinity adsorption on an insulin-agarose column caused depletion of insulin binding activity and immunoactivity to an identical degree. The latter results confirm the conclusion that the antibody binds to the insulin receptor molecule.

To further explore the antibody binding site (or sites) on the insulin receptor molecule, we exposed the receptor to trypsin at two concentrations known to impair the ability of the receptor to bind insulin (Fig. 2). After exposure to trypsin at concentrations of 40 and 200 μg/ml, receptor binding of insulin fell by 76 and 100 percent, respectively. However, the immunoassayable receptor concentration fell by only 29 and 57 percent, respectively. This demonstrates that the antibody (or one subset of antibodies in the serum) recognizes determinants on the receptor molecule distinct from those recognized by insulin and possibly removed from the actual insulin binding site. It is consistent with our previous observation (*8*) that trypsin treatment of adipocytes impairs insulin bioactivity



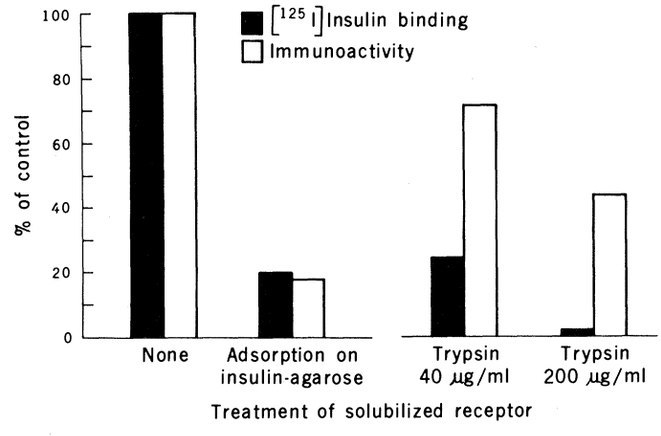


Fig. 1 (left). Radioimmunoassay of the insulin receptor. Solubilized human placental membranes (*10*) and [^{125}I]insulin (*15*) were prepared as previously described. [^{125}I]Insulin (2 ng; specific activity, 150 to 200 μCi/μg) was incubated with 3 ml of solubilized placental membranes in 0.1 percent Triton–0.1*M* sodium phosphate buffer at *p*H 7.4 (0.8 mg of protein per milliliter) for 1 hour at 22°C. The mixture was cooled to 4°C, then filtered over a Sephadex G-50 column (30 by 0.9 cm) at 4°C in the same buffer. The void peak of ^{125}I radioactivity was identified as labeled receptor by virtue of its precipitability by 12.5 percent polyethylene glycol (*10*), immunoprecipitability by several antibodies to the insulin receptor (*5*), and refiltration in the void volume at 4°C (*10*). In the immunoassay each tube contained 10 μl of a 1:20 dilution of antiserum B-4 to the receptor (immunoprecipitation titer, 1:4000) in a total volume of 750 μl containing 0 to 750 μl of unlabeled solubilized placental membrane in 0.1 percent Triton. After a 2-hour incubation at 22°C, the tubes were cooled to 4°C and 100 μl of [^{125}I]insulin-receptor complex (5000 to 7000 count/min) was added. After incubation for 2 hours at 4°C, 100 μl of sheep antiserum to human immunoglobulin G was added. After a further 2 hours at 4°C, immune complexes were precipitated in a Beckman Microfuge and washed once with 0.1 percent Triton buffer, and the radioactivity in the pellet was counted. The vertical axis shows the ratio of counts precipitated to counts precipitated in the absence of unlabeled solubilized receptor. The concentration of insulin binding sites had been determined previously by Scatchard analysis (*16*) of [^{125}I]insulin binding to solubilized membrane. The data shown for the placental receptor (squares) are means from three separate experiments, each performed in triplicate. Data for the mouse liver (triangles) and human IM-9 lymphocyte receptors (circles) represent duplicate determinations in a single assay. Details of the IM-9 lymphocyte studies are given in the legend of Table 1. Fig. 2 (right). Comparison of insulin binding capacity and immunoactivity of solubilized insulin receptor. The binding of tracer amounts of [^{125}I]insulin to solubilized placental membranes and the immunoactivity (see Fig. 1) of the same amount of material were each taken as 100 percent of the control value. Solubilized placental membranes (4.0 ml) in 0.1 percent Triton–0.1*M* sodium phosphate buffer were filtered twice over an insulin-agarose affinity column at 4°C. Portions (500 μl) of the column eluate were incubated with tracer amounts of [^{125}I]insulin to determine insulin binding and tested in the immunoassay as described. Solubilized placental membranes (1.0 ml in 0.1 percent Triton buffer) were incubated in the absence and in the presence of Trypsin (Boehringer, Mannheim, lot 1506433) at 40 and 200 μg/ml. After 15 minutes at 37°C, 10 μl of trypsin inhibitor (Sigma, lot 85C-8058; final concentration, 200 μg/ml) was added and the tubes were cooled to 4°C. Portions of the mixture were then tested for both [^{125}I]insulin binding and immunoactivity as indicated.

more readily than antibody-mediated insulin-like effects.

The immunoassay was used to study the nature of receptor down-regulation. Previous studies in our laboratory (*12*) showed that exposure of cultured human IM-9 lymphocytes to insulin results in a time- and temperature-dependent decrease in the insulin binding site concentration, determined from Scatchard analysis of labeled insulin binding. However, to determine whether the receptor is degraded, internalized, or simply "inactivated" in situ it is necessary to be able to measure the receptor independently of its binding function. To examine this question we produced maximum down-regulation of IM-9 lymphocytes by exposing them to $10^{-6}M$ insulin for 6 hours at 37°C. Insulin binding to the cells was decreased by 88 percent (Table 1). The cells were then solubilized in Triton X-100 and assayed for insulin binding (Fig. 3 and Table 1) and immunoreactive receptor (Fig. 1 and Table 1). As previously shown for intact cells (*12*), the Scatchard analysis shows a parallel leftward shift for the solubilized down-regulated cells, indicating a decrease in the apparent number of binding sites (Fig. 3, inset). However, the decrease in binding in the solubilized cells was only 62 percent (Table 1), suggesting dilution by intracellular binding sites, either preexistent, newly synthesized, or internalized. Triton extracts of both control and down-regulated cells produced displacements in the immunoassay along the same slope (Fig. 1). When expressed per milligram of protein, the immunoassayable receptor concentration in the down-regulated cells decreased 64 percent, identical to the decrease in binding site activity (Table 1). These data show directly that down-regulation of insulin binding is due to a decrease in the number of receptor molecules.

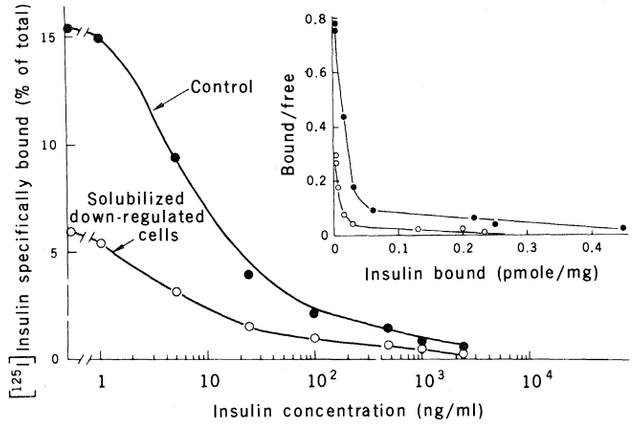


Fig. 3. Insulin binding to Triton-solubilized IM-9 lymphocytes after prior exposure of the intact cells to $10^{-6}M$ insulin for 6 hours at 37°C. Solubilized cell protein (2.4 mg) was incubated with 70 pg (19,000 count/min) of [^{125}I]insulin and increasing concentrations of unlabeled insulin in 200 μl of 0.1 percent Triton–0.1M sodium phosphate buffer for 90 minutes at 15°C. Receptor-bound radioactivity was precipitated in 12.5 percent polyethylene glycol (*10*). The inset shows the Scatchard plots (*16*) derived from the competition data.

We now consider the sensitivity of the immunoassay compared to that of conventional hormone binding studies. The current immunoassay can measure 0.1 nM insulin binding sites, a detection limit similar to that of direct binding studies (*13*). The sensitivity of binding studies is fixed and is determined by the affinity and cooperative properties of the hormone-receptor interaction. The sensitivity of the immunoassay depends on the affinity of the particular antibody for the receptor; appropriate selection of antibodies and manipulation of assay conditions might allow us to further improve sensitivity. Furthermore, the antibody-receptor interaction (*4*), unlike the insulin-receptor interaction (*14*), is very stable over a wide range of conditions of pH and ionic strength. The immunoassay should therefore be better suited to the detection of receptors in certain preparations such as tissue extracts and serum.

The ability to study the receptor molecule rather than just one of its functions (binding) has a number of potential applications. Our studies demonstrate the feasibility of measuring intracellular receptors and receptor turnover. The insulin binding site on the insulin receptor has been remarkably conserved throughout evolution. However, as indicated in the present study, even receptors within one species appear to differ immunologically. By using a variety of receptor antibodies it may be possible to study the receptor from a totally different perspective.

Finally, the assay may uncover new receptor diseases—those in which insulin binding is normal but the receptor is altered at other sites (post-binding site defects), or those in which binding is diminished or absent but the receptor concentration measured by immunoassay is normal. Radioimmunoassay of receptors should add a major new dimension to studies of these important membrane components.

Table 1. Down-regulation of the insulin receptor on IM-9 lymphocytes: quantitation by receptor binding and receptor immunoassay. The IM-9 lymphocytes were grown to late log phase in RPMI medium supplemented with 10 percent fetal calf serum. Cells (3×10^8) were sedimented by centrifugation at 500g, resuspended, and divided into two 50-ml lots. Insulin (0.3 mg; Elanco, batch 8KU3OD) was added to one lot (final concentration, $10^{-6}M$) and both lots were incubated for 6 hours at 37°C. The cells were sedimented and washed by resuspension for 30 minutes at 22°C in 40 ml of 0.1M sodium phosphate buffer, pH 7.4, containing 0.1 percent bovine serum albumin (BSA). This washing procedure was repeated three times to remove all bound insulin (*12*). Finally, each cell pellet was resuspended in 10 ml of Hepes [4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid] buffer, pH 7.8, containing 0.1 percent BSA. Binding was measured by incubating 1.5×10^6 cells with a tracer amount of [^{125}I]insulin (50 pg; 14,000 count/min) in 200 μl of Hepes buffer for 90 minutes at 15°C. Incubations were also performed in the presence of excess unlabeled insulin (10 μg per assay) to determine nonspecific binding. Cell-bound radioactivity was recovered by centrifugation in a Beckman Microfuge. The remaining cells ($\sim 12 \times 10^7$ per lot) were resuspended in 2 ml of 1 percent Triton–0.1M sodium phosphate buffer containing bacitracin (1000 U/ml), homogenized by hand for 3 minutes at 4°C, and incubated with stirring for 18 hours at 4°C. The mixtures were then centrifuged at 200,000g for 90 minutes. Portions of each supernatant ($\sim 6 \times 10^7$ extracted cells per milliliter) were assayed for both insulin binding activity (Fig. 3) and immunoreactive receptor (Fig. 1).

Cells	Intact cells	Triton-solubilized cells	
	Bound/free [^{125}I]insulin (per 10^6 cells)	Bound/free [^{125}I]insulin (per milligram)	Immunoassay, insulin binding sites (pmole/mg)
Control	0.202	0.078	0.080
Down-regulated	0.024	0.030	0.029
Decrease (%)	88	62	64

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13. Our estimate of assay sensitivity may depend on the validity of using a single-site cooperative model instead of a multiple-site model for insulin binding. If the multiple-site model were correct and the antibody bound only to high-affinity sites, our estimate of sensitivity obtained by extrapolation to the horizontal axis of the Scatchard plot (*11*) would be spuriously low. Thus, the estimated sensitivity presented here may be the minimum value.
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Transfer of Experimental Allergic Encephalomyelitis with Guinea Pig Peritoneal Exudate Cells

Abstract. *Incubation with specific antigen, myelin basic protein, greatly enhances the ability of guinea pig peritoneal exudate cells to transfer experimental allergic encephalomyelitis. Reproducibly successful transfers are obtained with 10^7 cells. With this relatively small number of cells, in vitro studies to determine the immunologic mechanisms involved in the disease process are now possible.*

Experimental allergic encephalomyelitis (EAE) is a cell-mediated autoimmune disease induced in susceptible animals by sensitization with central nervous system (CNS) myelin basic protein (BP) (*1*). Successful adoptive transfer of full-blown EAE in guinea pigs was originally reported by Stone (*2*), who found that transfer depended not only on viable histocompatible lymph node cells (LNC) but also required sensitization of donors with a large amount (2.5 mg) of mycobacteria in conjunction with whole spinal cord antigen. Falk *et al.* (*3*) confirmed these results and reported that EAE could also be transferred with cells from donors sensitized with large amounts of BP and mycobacteria (0.5 mg each). We later showed that a more reproducible transfer could be achieved (paralysis and CNS lesions in all recipients) by sensitization of donors with 0.5 mg of BP plus the larger amount of mycobacteria originally suggested by Stone (2.5 mg) (*4*). The number of LNC required for successful transfers was of the order of 10^9 regardless of whether whole tissue or BP was the sensitizing CNS antigen. On the basis of cell numbers, freshly harvested peritoneal exudate cells (PEC) are probably no better than LNC for disease transfer, since 5×10^8 PEC induced only mild disease in recipients (*5*).

Because of the large number of cells required, cellular transfer has not been extensively used for examining the specific immunologic mechanisms of EAE. We report here that in vitro culture of BP-sensitized PEC with specific antigen (BP) enhances the ability of PEC to transfer disease, reducing the number of cells required to induce both clinical and histologic signs of EAE in the recipients.

Male guinea pigs (strain 13) weighing 400 to 700 g (Division of Research Services, NIH) were used in all experiments. Donors were sensitized with either 100 μg of BP, 100 μg of BP plus 100 μg of ovalbumin (Sigma), or saline in 0.1 ml of complete Freund's adjuvant (CFA), containing 100 μg of H-37R$_v$, in a single intracutaneous site on the chest. Ten days after sensitization, the animals were injected in the peritoneum with 25 to 30 ml of sterile paraffin oil. Three days later the animals were anesthetized, and their peritoneal cavities were washed with 200 to 300 ml of Hanks balanced salt solution (HBSS).

After two washings in HBSS, the cells were suspended in RPMI 1640 medium containing 5 percent fetal calf serum, glutamine (300 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). These cells (2×10^7 to 4×10^7) were then plated in 100-mm plastic tissue culture dishes for 2 hours at 37°C in a humidified air mixture containing 5 percent CO_2. After incubation the nonadherent cells were removed, and the plates were washed once with medium. The pooled nonadherent cells were centrifuged and resuspended in medium. Viability was determined by trypan blue exclusion, and the number of phagocytic cells was determined by latex bead ingestion. All cell recoveries are based on viable cell counts. The cells were incubated for 72

Table 1. Transfer of EAE with cultured PEC.

Donor sensitization*	Culture	Antigen in culture		Cells (10^7)	No. sick / No. recipients	D.I.† (mean ± S.E.)
		Concentration (μg/ml)	Type			
100 μg of BP + CFA	−			4.0	0/2	0
	−			2.0	0/1	0
	+	10	BP	4.0	6/6	8.4 ± 0.6
	+			3.5	2/2	9.0 ± 0.0
	+			2.0	5/5	9.1 ± 0.6
	+			1.0	10/10	8.0 ± 0.4
	+			0.5	3/6	8.0, 9.0, 7.5‡
	+			0.25	2/4	8.0, 9.0‡
	+			0.1	2/3	6.0, 9.0‡
100 μg BP + 100 μg Ova + CFA	+	None		3.0	0/1	0
	+			2.0	0/1	0
	+	10	BP	2.0	1/1	7.5
	+			1.0	1/1	9.0
	+			0.6	1/1	7.5
	+	100	Ova	4.0	0/1	0
	+			2.0	0/1	0
CFA	+	10	BP	4.0	0/2	0

*Myelin basic protein was isolated from guinea pig CNS tissue (*10*) and purified by ion exchange chromatography (*11*). †D.I. = Disease index—severity of disease based on a combination of clinical and histologic signs of disease (*6*). ‡Disease indices of sick animals.