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RECEPTOR LOCALIZATION AND DISTRIBUTION ON NORMAL
AND DIABETIC CELLS BY ELECTRON MICROSCOPY

by

Vance L. Kincaid, II

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
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Western Michigan University
Kalamazoo, Michigan
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RECEPTOR LOCALIZATION AND DISTRIBUTION ON NORMAL
AND DIABETIC CELLS BY ELECTRON MICROSCOPY

Vance L. Kincaid, II, M.S.

Western Michigan University, 1988

The objective of this research was to evaluate receptor localization and distribution on normal and diabetic cells by electron microscopic analysis.

To achieve this objective, gold-adsorbed and ferritin-conjugated macromolecules were used to determine the location and concentration of specific receptor binding sites on the cells surfaces. To facilitate the use of these ultra-structural histocytochemical markers, normal and diabetic Chinese hamster and other nondiabetic cells were either grown in vitro on ultra thin plastic films or embedded in Lowicryl K4M and pre- or post-labelled with these marker molecules.

Analysis of the data obtained from electron micrographs of these labelled cells was used to determine the densities and distributions of the various diabetic-related markers on the cells surfaces.

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Vance L. Kincaid, II

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by electron microscopy**

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Western Michigan University, 1988

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CHAPTER I

INTRODUCTION

The purpose of this research was to investigate receptor localization and distribution on normal and diabetic cells utilizing electron microscopy. In order to accomplish this, a number of experimental techniques were developed and existing methodologies modified. The Chinese hamster was chosen as the animal model because these animals were specifically developed by The Upjohn Company of Kalamazoo, Michigan to mimic the human conditions of diabetes.

Evaluating the role of peptide hormones and their cellular receptors is the first step in understanding the mechanisms of diabetes. Through the use of various ultra-structural techniques via cytohistochemical markers to visualize membranes in diabetic cells, we hoped to determine whether any generalized ultrastructural abnormality exists in their receptor processes. This may then elucidate a more primary cause of diabetes dysfunction.

This research is not about elucidating the mysteries of diabetes but the methods at the ultrastructural level

to determine if there are differences in receptors' patterns between normal and diabetic cells. However, a brief background on diabetes, the molecular mechanism of insulin action, and the structure and function of the insulin receptors follows.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Diabetes

Diabetes mellitus and its complications are now thought to be the third leading cause of death in the United States (Bruckel, 1985; Siwolop, 1984). It is an ancient disease. The earliest description of its symptoms is found in the Ebers papyrus of Egypt, dating back to 1500 B.C. In the second century A.D. Aretaeus of Cappadocia named the disease diabetes, which is the Greek word meaning "to flow through a siphon." In the sixth century Indian physicians recognized that urine from diabetic patients had a sweet taste. But it was not until the 18th century that the sweet-tasting substance was recognized as glucose and the word mellitus, or "honeyed," was added (Notkins, 1982).

There are two types of diabetes: Type I and Type II. Type I is also known as juvenile-onset or insulin-dependent (IDDM). Type II is also known as maturity-onset or non-insulin-dependent (NIDDM). Differences in Type I and II are summarized in Table 1. Type II is commoner and not as severe as Type I. Both Type I and II

Differences Between Type I and Type II Diabetes

Features	Type I	Type II
Age at onset	Usually under 20	Usually over 40
Proportion of all diabetics	< 10%	> 90%
Seasonal trend	Fall and winter	None
Appearance of Symptoms	Acute or subacute	Slow
Metabolic keto-acidosis	Frequent	Rare
Obesity at onset	Uncommon	Common
Beta cells	Decreased	Variable
Insulin	Decreased	Variable
Inflammatory cells in islets	Present initially	Absent
Family history of diabetes	Uncommon	Common
HLA association	Yes	No
Antibody to islet cells	Yes	No
Receptor function	Normal	Abnormal
Cause	Environmental?? Other??	Affluence/obesity?? Other??

diabetes prevent the body from using food that the digestive process converts into glucose and amino acids. The sugar accumulates in the blood stream instead. But the two forms of the disease differ vastly in their biochemistry. In Type I, enough or normal chemical receptors are usually present on the cell surfaces to receive insulin, the peptide hormone that delivers the glucose to the cell which turns it into energy. The problem is that the body itself produces no insulin and therefore requires daily injections of insulin. In Type II diabetes, the body usually manufactures enough insulin but cannot respond to it properly. Some Type II victims, especially those in an early stage of the disease, have too few receptors on the surfaces of their cells (Sanders, 1981). In other cases, particularly in more advanced ones, a breakdown in the cell machinery appears to prevent the glucose from entering the cell to be burned as energy. In both early and advanced forms of Type II, the major cause is thought to be the same: obesity. As fat cells increase in size, the number of chemical receptors on their surfaces decreases and the defects within the cell cause glucose metabolism to go awry.

In both Type I and II, the results are the same. Failure to make or respond to insulin causes glucose levels in the bloodstream to rise to dangerously high

levels, setting off a chain reaction that, if unchecked, can eventually lead to coma or death. However the exact cause(s) of diabetes remains unclear (Bruckel, 1985; Notkins, 1982; Siwolop, 1984). But, a defect anywhere along the pathway from insulin synthesis to the peripheral action of insulin could result in diabetes. Possible causes include: (a) destruction of pancreatic beta cells, (b) abnormal synthesis of insulin, (c) retarded release of insulin, (d) inactivation of insulin in the bloodstream by antibodies or other blocking agents, (e) altered insulin receptors or a decreased number of receptors on peripheral cells, (f) defective processing of the insulin message within the target cells, and (g) abnormal metabolism of glucose.

Insulin: Molecular Mechanism of Action

Regulation of cellular metabolism and growth by insulin is a result of a series of events initiated by the interaction of the hormone with specific cell surface receptors. Figure 1 describes the normal role of insulin. Insulin, secreted by the pancreas into the blood stream, becomes attached to a target cell, via a specific surface receptor, to help metabolize glucose, among other functions. The rise in blood glucose associated with a carbohydrate meal induces the pancreatic beta cells in the islets of Langerhans to

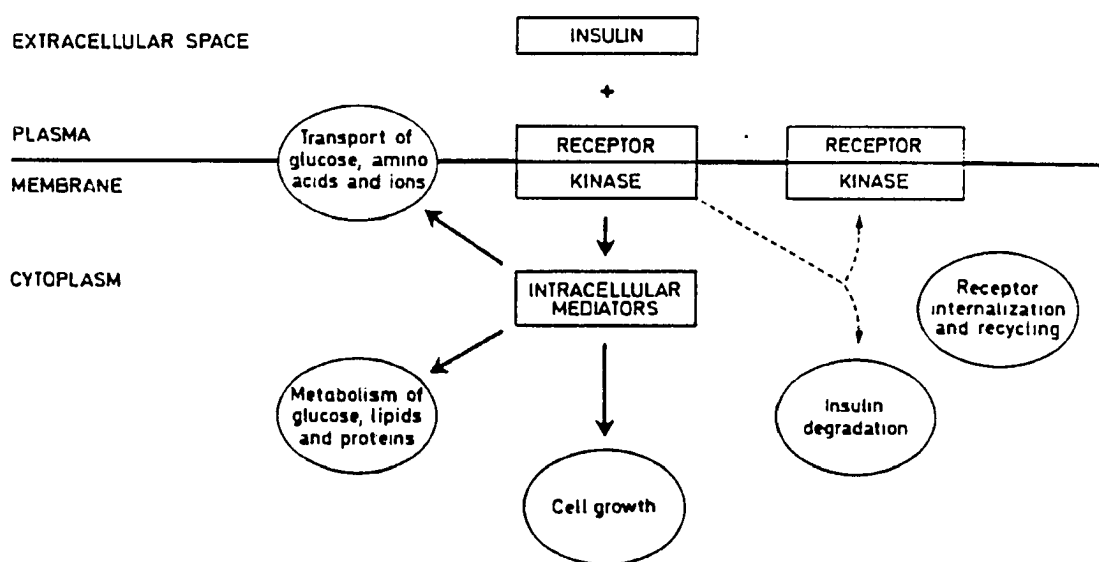


Figure 1. Cellular Mechanism of Insulin Action
(Van Obberghen & Gammeltoft, 1987).

secrete insulin into the circulation. There are approximately 3000 beta cells per islet (Orci, Vassalli, & Perrelet, 1988) The insulin is then carried in the bloodstream to target cells throughout the body, where it binds to receptor molecules on the cell surface. This interaction triggers a series of events inside the cells that enhance the uptake of glucose from the blood and its subsequent breakdown for metabolic energy or storage as glycogen (animal starch) and storage and/or breakdown of fat.

The physiological effects of insulin in mammalian systems include stimulation of hexose, ion, and amino acid uptake; modification of the activities of rate-limiting enzymes such as glycogen synthase and hormone-sensitive lipase by net dephosphorylation; phosphorylation of seryl residues in proteins such as adenosine triphosphate (ATP); regulation of gene expression for a small number of regulatory enzymes; redistribution of membrane proteins such as the glucose transporter and the insulin-like growth factor II (IGF-II) and transferrin receptors; and promotion of cell growth. Many of these effects are tissue- or cell-specific and involve only a discrete subset of proteins.

Table 2 shows that the chronology of insulin action varies. Transcription of the gene encoding phosphoenolpyruvate carboxykinase is inhibited within

Chronology of Insulin Action

Time	Event
Seconds	Binding to receptor Activation of receptor protein tyrosine kinase Receptor autophosphorylation
Seconds to minutes	Changes in gene transcription Stimulation of hexose and ion transport Ligand-mediated receptor internalization Alterations in intracellular enzyme activities Seryl and threonyl phosphorylation of the receptor
Hours	Synthesis of protein, lipid, and nucleic acid Maximal down regulation of the receptor Cell growth

Source: Rosen, O. M. (1987).

seconds of addition of insulin, whereas growth promotion requires hours of exposure. Many of the rapid actions of insulin, such as stimulation of hexose transport and alterations of enzyme activities, do not depend on synthesis of new proteins or nucleic acids. Even though this is an incomplete summary, the processes initiated by the actions of insulin invokes seryl and threonyl phosphorylations and dephosphorylations of cytosolic and mitochondrial proteins, membrane translocations with probable cytoskeletal protein involvement, and nuclear action.

It is thought that a single mechanism is involved in initiating all of the normal biological effects with the first essential and common step in insulin action, interaction with the insulin receptor. It is at the receptor level that this research is directed. But in order to investigate this, electron-opaque markers were synthesized to locate these binding sites at the ultrastructural level.

Insulin Receptor: Structure and Function

Fifty years after the discovery of insulin its cellular mechanism of action (i.e. the events following receptor binding and leading to the ultimate cellular responses) still remains unclear (Rosen, 1987; Wajchenberg & Lerario, 1988). Recent progress in the

molecular characterization of the insulin receptor pertinent to the mechanism of insulin action include: (a) the discovery that the insulin receptor is an insulin-dependent protein tyrosine kinase (activated by autophosphorylation of the insulin receptor) functionally related to certain growth factor and oncogene-encoded proteins, (b) the molecular cloning of the insulin proreceptor complementary DNA (cDNA), and (c) evidence that the protein kinase activity of the receptor is essential for insulin action (Carpentier, Gordon, Robert, & Orci, 1987; Rosen, 1987; Van Obberghen & Gammeltoft, 1987).

The insulin receptor, an integral plasma membrane glycoprotein with intrinsic enzymatic activity capable of selectively binding insulin with high affinity, is the necessary first step in insulin action. The insulin receptor is ubiquitously distributed in mammalian organisms with cells expressing different numbers of receptors ranging from 10^2 to 10^5 per cell. The basic oligomeric structure of the receptor is the same in all vertebrates examined. The receptor is a heterodimer composed of four glycosylated subunits, with relative molecular mass (M_r) of ~350,000 daltons (Figure 2). The two alpha subunits, M_r ~130,000, bind insulin and are linked to each other and the two beta subunits, M_r ~95,000, by disulfide bonds. The disulfide bonds linking

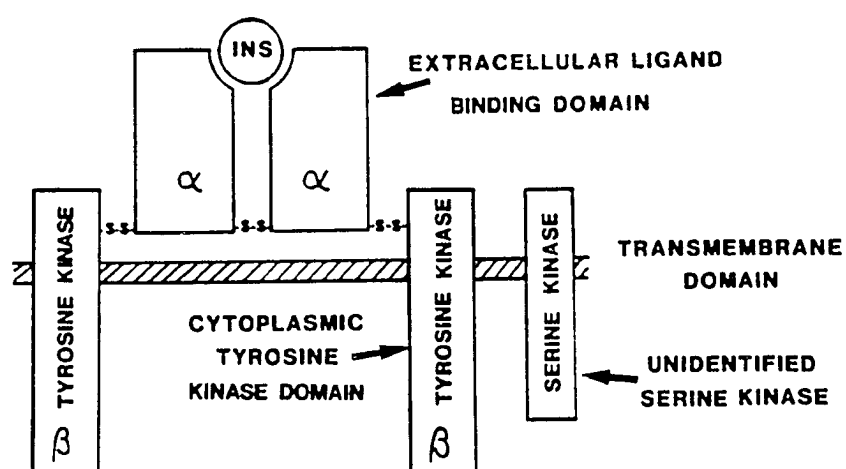


Figure 2. Schematic Model of the Insulin Receptor Kinase Complex (Van Obberghen & Gammeltoft, 1987).

the two alpha-beta dimers are more sensitive to reduction than those linking the alpha and beta subunits to each other. Thus the functional cell surface receptor is composed of two functional domains, one with binding activity and another with tyrosine-specific protein kinase activity capable of undergoing autophosphorylation. There is a serine kinase noncovalently associated with the receptor which is thought to play a role in insulin's metabolic actions, whereas the tyrosine-specific enzyme activity is involved in insulin's growth-promoting action (Van Obberghen & Gammeltoft, 1987). In addition insulin binds to and promotes phosphorylation of the insulin receptor precursor (proreceptor) a monomeric protein of Mr ~210,000.

The independant alpha and beta subunits are synthesized by way of this high molecular weight single chain precursor and are a product of a single gene on chromosome 19 (Carpentier et al., 1987). This proreceptor is cleaved and further processed by addition of complex carbohydrates prior to insertion into the plasma membrane as the insulin receptor kinase complex. It is thought that the biosynthesis of the insulin receptor occurs in the same fashion as other integral membrane glycoproteins involving the nucleus, the rough endoplasmic reticulum and the Golgi apparatus. Further,

there must be some as yet unidentified form of transport of the mature receptor subunits to the plasma membrane where they are inserted.

The half life of the insulin receptor, estimated at 7-12 hours, is shortened by exposure of the cell to ligand. The latter phenomenon, a component of down regulation or net loss of cell surface receptors on exposure to the homologous ligand, is thought to play a role in enabling cells to regulate their sensitivity to ambient concentrations of hormone (Carpentier et al., 1987; Rosen, 1987). In a separate series of events the insulin receptor complex can be removed from the cell surface by receptor mediated endocytosis. The endocytic route involves formation of coated pits, coated vesicles, large clear vesicles or endosomes, multivesicular bodies and other lysosomal forms or an as yet unidentified organelle required in recycling receptors. Currently, with respect to the insulin receptor, the biosynthetic pathway and the endocytic pathway appear to be separate and do not exert any regulatory function over each other. There is however conflicting data over coated versus uncoated pinocytotic vesicles for insulin receptor cycling (Moll, Thun, & Pfeiffer, 1986; Smith & Jarett, 1988).

The biological relevance of insulin receptor phosphorylation is not yet clear. It is thought that it

plays a role in cellular processes such as receptor affinity regulation, hormone and receptor internalization and signal transmission. These phenomena are well-characterized in current literature but their molecular mechanism is almost completely unknown. It is thought that receptor regulation and transmembrane signaling are integrated events in insulin action and the process of receptor autophosphorylation is involved in transmission of the insulin message to cellular enzymes and transport carriers. It is postulated that covalent receptor modification is an early step in insulin action and that the increased kinase activity of the insulin receptor evoked by hormone binding would lead to phosphorylation-dephosphorylation of other cellular proteins, and through the generation of a cascade of reactions this would result in the final effects of insulin. Conditions with cellular resistance (Type II diabetes) are coupled with decreased phosphorylation of the insulin receptor. This evidence indicates a molecular defect is involved in the disease (Van Obberghen & Gammeltoft, 1987).

The existence of the insulin-insulin receptor system is based on accumulating information about the biochemical, physiological, and pathophysiological consequences of insulin action. This information can be used to trace the distal actions of insulin (e.g., dephosphorylation of glycogen synthase or phosphorylation

of ribosomal protein S6) back to the earliest reactions initiated by the binding of insulin to its receptor. Pathways may be analyzed in normal cells and compared with those in insulin-resistant cells derived from patients with diabetes mellitus or from animal models of obesity and diabetes.

Basically two kinds of genetic systems are available for studying insulin action. The first are human or animal diabetic models in which the disease has a heritable component. Some forms of the disease result from deficiencies of insulin secretion or aberrancies in insulin structure and processing. In other forms of diabetes there are abnormalities in insulin receptor content, insulin binding, or receptor protein tyrosine kinase activity. It is thought also that some diabetics have defects in proteins with which the receptor interacts. Currently it is possible to delineate the molecular alterations in the receptor gene that result in abnormal receptor content or functional defects in receptor structure. Many if not all of these defects ultimately manifest themselves as cellular deficiencies in insulin-dependant protein kinase activity. There is speculation that new approaches to therapy may evolve from an understanding of the critical differences between this enzyme and other types of protein kinases (Rosen, 1987).

A great deal of knowledge about the early actions of insulin has been generated over the last five years but why insulin does not bind or what happens after it binds remains incomplete. However the use of electron-opaque markers for binding and intracellular tracing are providing insight into insulin-insulin receptor processes (Csaba, Madarasz, & Bohdaneczky, 1987; Cunningham, Wolken, & Ackerman, 1984; Moll et al., 1986; Smith, Goldberg, & Jarett, 1988).

Electron-Opaque Markers

Cytochemical marking of cell surface and intracellular components for microscopical observation is an extremely important technique for studying molecular organization and cell function. Biochemical data alone would be incomplete since the data obtained represent average values and give little information regarding the distribution of cellular components. A large number of cytochemical techniques have been developed in order to identify, localize, quantify, and understand the dynamics of cell components at the ultrastructural level.

Besides freeze-etching, X-ray microanalysis and the occasional use of markers such as haemocyanin, all other cytochemical methods for transmission electron microscopy (TEM) including autoradiography depend upon reactive products opaque to the electrons or on the use of

electron-opaque particulate markers. The latter was used in this research in the form of ferritin-insulin (F-I) and gold-albumin-insulin (GAI).

These methods can be classified into different categories:

1. They have a broad specificity (e.g., cationic ferritin) or a narrow specificity (e.g., colloidal gold, ferritin, peroxidase conjugates).

2. The methods are general (e.g., colloidal gold, ferritin, labelled and unlabelled peroxidase conjugates, avidin-biotin complexes) or restricted to particular cases (e.g., glycosylated ferritin and peroxidase).

3. Markers are diffuse (e.g., peroxidase conjugates) or particulate (e.g., ferritin, colloidal gold).

4. Conjugates are obtained via non-covalent bonds (e.g., colloidal gold), covalent bonds (e.g., ferritin and peroxidase conjugates) or specific interactions (e.g. peroxidase-antiperoxidase [PAP], avidin-biotin complexes).

5. Marking is achieved in one step (direct) or in multiple steps (indirect).

6. Some methods can be quantified (e.g., colloidal gold, ferritin). Although most of these markers have found application in TEM (pre- and post-embedding

techniques), only a few have been developed for scanning electron microscopy (SEM).

In TEM, for each localization problem, choices have to be made concerning: (a) the preparation of the material under study, (b) the marker, (c) the identifier and/or linker, and (d) the marking procedure. The "appropriate" method will be largely determined by the kind of information and the degree of resolution desired. For instance, the localization of cellular components requires balanced fixation of the cell under study because antigenic determinants must not be denatured and the ultrastructural morphology of the cell should be well preserved. These two requirements tend to be mutually exclusive. However the recently introduced low temperature embedding procedure has resulted in enhancement of structural preservation and immunocytochemical labelling, especially with the protein A-gold method (Roth, Bendayan, Carlemalm, Villiger, & Garavito, 1981; Roth, 1982c). Alternatively, cryoultramicrotomy allows optimal preservation of ultrastructure and immunoreactivity due to mild fixation and the possibility of avoiding dehydration steps (Tokuyasu, 1980).

Because of the number of methods available and the variety of possible combinations, immunocytochemists experience difficulties in making the proper choice to

solve a specific problem. Useful criteria of selection are: specificity, sensitivity, easiness and cost of techniques (time and money-wise). Ideally, markers should be easily recognized and quantified. They should also be readily prepared from a variety of molecules recognizing ligands with a narrow specificity. Although diffuse markers are generally sensitive since they are based on amplification effects, particulate markers are especially convenient for precise localization and quantification.

Particulate Markers

Colloidal Gold

A considerable advance in the understanding of the behavior of colloidal systems was put forth by the DLVO theory (Deryagin and Landau, 1941; Verwey and Overbeek, 1948). In this theory, the particle is considered to consist of two components: one arises from the overlap of the electrical double layer and leads to repulsion, and the other from electromagnetic effects which leads to Van der Waal attraction. When a colloidal particle approaches a surface, the potential energy of interaction is as follows: At some finite distance, when the surface does not come into molecular contact, an equilibrium is reached between attractive and repulsive forces (secondary minimum, reversible adhesion). At a smaller

distance, a net energy barrier occurs. Once this is overcome, the theory predicts, another minimum occurs (primary minimum, irreversible adhesion). Both the height of the energy barrier and secondary minimum depend on ionic strength and electrostatic charge. Although it is still generally believed that the coagulation concentration of metal colloids in the presence of electrolytes is independent of particle size, Frens (1972) has shown that the stability of metal colloids depends largely on size. For instance, small gold particles are more stable against electrolyte coagulation than coarser suspensions. These are explained by the diminished Van der Waal attraction between smaller particles.

Gold particles carry a net negative charge which causes mutual repulsion and as a consequence, stability of the colloid. The addition of electrolytes results in a compression of the ionic double layer surrounding the particles. As a result, the colloid will coagulate. However, coagulation can be prevented when a protective coat is added onto the particles by merely mixing with a solution of macromolecules. This is the simple principle on which the preparation of all gold markers is used.

The first application of colloidal gold as TEM specific immunocytochemical marker was described in 1971

by Faulk and Taylor. The method was then introduced by Horisberger, Rosset, and Bauer for SEM in 1975.

For TEM and SEM application, monodisperse gold colloids are prepared essentially by three procedures: The smaller particles, Au₅ (the subscript indicates the mean diameter of the particles in nm) are obtained by reducing gold chloride (chloroauric acid, HAuCl₄) with yellow or white phosphorus (Faulk & Taylor, 1971; Horisberger & Rosset, 1977). Au₁₂ particles are produced in the presence of sodium ascorbate as the reducing agent (Horisberger & Tacchini-Vonlanthen, 1983b). For Au₁₆ to Au₁₅₀ particles, the reducing agent is sodium citrate (Horisberger, 1979; Horisberger & Rosset, 1977). For the latter preparation the smaller the particle size, the higher the concentration of the reducing agent.

Colloidal gold has been labelled with a variety of molecules such as toxins, hormones, polysaccharides, glycoproteins, proteins such as protein A, enzymes, lectins, immunoglobulins, lipoproteins (cited by Horisberger, 1981b). The list is still expanding. Recently the avidin-biotin complex has been added. With small molecules, difficulties have been experienced in stabilizing colloidal gold (Horisberger, 1981b). In this case, stable markers can be obtained when the labelling molecules are cross-linked to a carrier molecule such as bovine serum albumin (Horisberger & Rosset, 1977).

Detailed information on the preparation of gold markers has been published by Horisberger and Rosset (1977) and by Geoghegan and Ackerman (1977).

Much evidence indicates that macromolecules, adsorbed onto gold particles as a monolayer presumably through non-covalent binding process, remain firmly attached and keep their bio-activities for months or even years (Horisberger, 1981b; Horisberger & Tacchini-Vonlanthen, 1983a).

In TEM Au₅ to Au₂₀ particles are commonly used. The selection of the size is based on a compromise between the degree of magnification necessary and the density of the marking obtained. As a rule, the density of marking increases when the particle size decreases. Due to their electron opacity and characteristic shapes, gold particles are easily recognized, even on post-stained sections. Both the direct and indirect methods are used. The protein A-gold method popularized by Roth (1982c) and other groups have found numerous applications and gives excellent results especially in combination with embedding at low temperature (Roth et al., 1981) to retain as much as possible the antigenicity of intracellular proteins.

When post-embedding techniques were compared (Horisberger, 1981a) the density of marking observed was

direct lectin < direct antibodies < indirect antibodies < indirect protein A.

The colloidal gold method is also suitable for multiple marking of cell surface and intracellular components since the colloids are available in different sizes (Horisberger, 1979,1981b).

Ferritin

A vast number of investigations have made use of ferritin as an electron-dense marker for TEM (Jarett, Schweitzer, & Smith, 1980; Jarett & Smith, 1977; Morgan, 1972; Nicolson & Singer, 1971; Smith & Jarett, 1981; Sternberger, 1979). Ferritin with a molecular weight of 750,000 has a protein shell of about 12nm outer diameter surrounding an inner core of ferric hydroxide micelle (5.5-6.0nm in diameter) containing more than 2,000 iron atoms per molecule. The first conjugate of ferritin was made by the covalent coupling of antibodies (Rifkind, Hsu, Morgan, Seegal, Knox, & Rose, 1960; Singer, 1959). Ferritin-antibody conjugates are widely used and permit the localization of specific components in biological specimens with a resolution of about 30nm (Singer & Schick, 1961).

The method is general for TEM application but cannot yet be resolved by most scanners for SEM. Both the direct and the indirect techniques are used. Indirect

techniques are based on the use of conjugates such as ferritin-protein A (Templeton, Douglas, & Vail, 1978), ferritin-avidin (Heitzmann & Richards, 1974), and biotinylated ferritin (Bayer, Skutelsky, & Wilchek, 1979).

Conjugation of protein to ferritin is carried out by using glutaraldehyde, meta-xylene diisocyanate or toluene 2,4-diisocyanate as coupling agents. The preparation of the conjugate has been described step by step by Hsu (1981). The conjugates must be freed of unconjugated protein and ferritin. $F(ab')_2$ fragments which exhibit a similar affinity to that of IgG can be used for the preparation of the conjugates. The removal of the F_c region of the molecule reduces non-specific binding and decreases steric hindrance (Kraehenbuhl & Jamieson, 1974).

Several difficulties have been encountered in the use of ferritin conjugated to antibodies. Conjugates generally exhibit reduced antibody activity relative to the original antiserum. Therefore the use of highly purified ferritin and specific antisera of high titre is essential. Ferritin particles are not easily recognized on negatively stained thin sections. In post-embedding techniques, non-specific adsorption is often difficult to eliminate. This is due to the fact that ferritin, which is negatively charged at neutral pH, tends to bind

tightly to several internal structures. Several approaches have been used to improve marking specificity (Parr, 1979) and increase electron opacity (Ainsworth & Karnovsky, 1972).

Choice of Procedure

It is often difficult to select the correct method to solve a specific problem since few comparative studies are available in this field of research.

As a rule, the density of marking achieved is superior with indirect procedures since, in principle, several markers can labell a single site and hence provide an amplification effect. However, indirect methods require several incubation steps which may adversely affect cell surface morphology. The first bound ligands may also be lost to a certain extent during washing steps. Occasionally, indirect methods have been found to be less sensitive than direct procedures (Briggman & Widnell, 1983). When shedding is unavoidable even after fixation, indirect methods are to be preferred since the first ligand applied generally binds rapidly to the cell surface and stabilizes the structure (Horisberger and Vonlanthen, 1979b). Finally, a high density of marking with particulate markers is not always beneficial since the fine organization of the cell structure may be obscured.

Among the commonly used markers, ferritin and gold particles seem to provide the highest resolution. However, colloidal gold stains may not identify all reactive sites in the post-embedding technique (Childs, 1983b). With frozen thin sections, the protein A-gold method (two steps) was found to be superior for localizing antigens when compared to ferritin and peroxidase conjugates (one step) (Beesley, Orpin, & Adlam, 1982). When the sensitivity of ferritin conjugated to goat anti-rabbit immunoglobulin (two steps) was compared to the protein A-Au₅ method (two steps), the number of ferritin particles per unit area was more than three times that of the gold particles. However, using a three-step fold method, staining increased to the level of the two-step ferritin procedure (Tokuyasu, 1983).

Besides density of marking and sensitivity, steric hindrance is another factor to consider. Although enzyme conjugates are not devoid of steric hindrance, particulate markers are subject to it especially when pre-embedding procedures are used. Unless the size of particulate markers is varied, false-negative results are difficult to detect.

Contrary to diffuse markers, particulate markers are easily amenable to quantification either by counting the bound particles (ferritin, colloidal gold) or in a pre-embedding procedures by spectrophotometric measurements

(colloidal gold). Quantitative data should be critically examined since the density of marking depends on several factors such as embedding medium, fixative used, and size of the markers.

Finally, if one wishes to achieve double marking, a number of possibilities exist. Pairs such as gold and peroxidase conjugates, gold particles and ferritin conjugates, iron-dextran and ferritin conjugates are compatible. The gold method is particularly well suited for multiple marking in pre- and post-embedding procedures since particles of different sizes are available (Horisberger, 1979; Horisberger & Rosset, 1977). Double immunogold procedures are considered in more detail by Polak and Varndell, (1984, pp. 155-177).

CHAPTER III

MATERIALS AND METHODS

Chinese Hamsters

Two late stage diabetic animal sublines, AC and AH, developed by The Upjohn Company were used with a normal control M line. The AC subline has a low level of circulating insulin (insulinopenic) and high levels of glucose (hyperglycemic) and glucagon (hyperglucagonic). The AH subline has a normal level of circulating insulin and also has high levels of glucose and glucagon. The M line has normal levels of circulating insulin, glucose and glucagon. The late stage AH subline animals (12-16 months of age) have overt hyperglycemia and ketoacidosis. The AC subline has some similarities to Type I diabetes in humans and the AH subline has some similarities to Type II diabetes in humans.

Chinese Hamster Peritoneal Macrophages

The animal was killed by cervical dislocation. The abdominal skin was then wet with 70% ethanol (EtOH) and the peritoneal cavity massaged for one minute. The skin over the peritoneal cavity was retracted and 10ml of 37°C

minimum essential medium (MEM) with 1% fetal calf serum (FCS) and 10 units/ml nonpreserved heparin was forced into the cavity then a volume was pulled back into the syringe and forcefully reinjected. This lavage procedure was repeated two times. The fluid was then recovered from the animal and the macrophages dispensed over the desired substrate. The substrate that the cells were planted on was formvar-coated glass slides in nonwettable petri dishes or on formvar-coated glass petri dishes. After planting the cultures were gased (5% CO₂ in air) and incubated at 37°C for one hour. Immediately following the culturing of the macrophages other tissues of interest for this study were removed from the animal (e.g. liver, pancreas, kidney, muscle and fat).

The tissues were minced into approximately 1mm³ pieces then minced further into <1mm³ pieces and immediately placed in 10ml of fixative at 0°C. The fixative was 2% paraformaldehyde-0.2% glutaraldehyde (PG) in 0.1M cacodylate buffer pH 7.4 with 0.2M sucrose. The tissues were then held in this fixative at 4°C and later embedded in Lowicryl K4M.

After the initial incubation period the macrophage cultures were washed three times in 37°C Hanks balanced salt solution (HBSS) (or serum free MEM) to remove nonadherent cells and then fed with MEM (with 10% FCS and antibiotics) regassed and again incubated at 37°C for two

hours. The formvar substrate cultures was then washed free of growth medium with 37°C HBSS, drained and processed by the methods for the marker systems employed in this study.

Tissue

Tissues of interest were immediately removed from the animal (e.g. liver, pancreas, kidney, muscle and fat) and placed in cold (0°C) fixative. The tissues were then minced into ~1mm³ pieces and rinsed in fixative, then minced further into <1mm³ pieces, rinsed several times more, then held in fixative at 4°C for further processing and embedding.

Fixative

Paraformaldehyde-glutaraldehyde (PG) and paraformaldehyde-glutaraldehyde-picric acid (PGPA) gave better ultrastructural preservation for EM than either aldehyde alone. Table 3 lists the concentrations of PG and PGPA used for fixation.

Thin Film

The support substrate was a new 50mm glass petri dish washed in acetone then absolute alcohol. The plastic substrate was made from 0.5g of formvar resin

dissolved in 99.5ml ethylene dichloride. The bottom of the petri dish was flooded with 1.0ml of the 0.5% formvar

Table 3

PG and PGPA Fixative Concentrations

PG	PGPA
2% paraformaldehyde	same
0.2% glutaraldehyde	same
0.2M sucrose	same
1mM MgCl ₂	same
1mM CaCl ₂	same
	0.02% picric acid
in 0.1M sodium cacodylate buffer pH 7.4.	

solution then the excess was immediately withdrawn with a Pasteur pipet. The plate was set on a level surface for several minutes to dry. The cover was then replaced on the petri dish slightly ajar and sterilized at 70°C for 4.0 hours to overnight or with UV light. A smooth almost invisible sheet of film remains.

Cells were then transferred to this coated surface and allowed to grow to confluency. Once a desired amount of cell growth occurred, conventional EM procedures were followed (i.e. fixation, labelling, dehydration, etc.). After alcohol dehydration the entire sheet of film was released intact from the petri dish by addition of

propylene oxide mixed 1:1 with ethylene dichloride. The sheet released after 10-15 minutes with gentle swirling or proding with a rubber policeman. Once free the sheet of cells was gently lifted with forceps which condensed it into a tightly folded mass or the sheet was placed in a conical micro tube and spun into a pellet which allowed for ease of handling during embedding steps.

Preparation of Monomeric Ferritin-Insulin (F-I)

The reaction was carried out in a polypropylene beaker by dissolving 5mg of insulin in 1ml of 0.05M KCL-HCL, pH 2.0, followed by an additional 4.5ml of KCL-HCL buffer and 75mg of ferritin monomer. The mixture was stirred for 3-5 minutes at 24°C and four 125ul aliquots of a 1:75 dilution of 50% glutaraldehyde was added at 10 minute intervals with constant stirring. The mixing was continued for 60 minutes at 24°C and an additional 90 minutes at 4°C. The reaction was stopped by adding 150ul of 1M lysine-HCL and stirring for 30 minutes at 24°C. Any aggregates were removed by a 10,000g centrifugation. The supernatant was collected. The ferritin and ferritin-insulin and free insulin were neutralized by slow addition of 1N NaOH to pH 6.0 and 0.1N NaOH to pH 7.4. If the solution turned muddy brown between pH 5.5 and 6.5, the pH was raised to 8.0 and back titrated to pH 7.4 with 0.1M phosphate buffer which provided a clear

ruby colored solution. The resulting solution was dialyzed to equilibrium with 50mM sodium phosphate pH 7.4. The monomeric ferritin-insulin was collected and concentrated by centrifugation at 150,000g for 2 hours. The F-I was purified further by passing it through a Sephadex G-100 column (1x55cm) and concentrating it once again by ultracentrifugation. The pellet was resuspended in 0.01M sodium phosphate buffer and stored at 4°C but never frozen. An O.D. 440 indicated that F-I had a concentration of 1.7mg/ml. The molar ratio of ferritin to insulin (F/I) was 4.5/1. The insulin RIA revealed 1.4U/ml of insulin activity or 20ug/ml of insulin.

F-I Histochemical Procedure

Cells were normally incubated with F-I label for up to 1 hour at room temperature. Controls consisted of cells or thin sections incubated with no label, free insulin, free ferritin, and free insulin followed by F-I.

Preparation of Insulin-Bovine Serum Albumin Conjugate

An excess of porcine insulin (2mg) and globulin-free bovine serum albumin (5.2mg) were dissolved in 0.5ml of 0.01N NaOH and the pH adjusted to 7.0 with 0.01N HCL. To this solution was added 0.1ml of 0.25% glutaraldehyde. The conjugation reaction proceeded for 2 hours at room temperature. The reaction mixture was concluded by the

addition of 24.5ml of 0.005M NaCl and the final solution filtered through a 0.45u filter.

Preparation of the Insulin-Bovine Serum Albumin-
Colloidal Gold Complex (GAI)

Colloidal gold, pH 5.3, was prepared as outlined by Geoghegan and Ackerman (1977). Colloidal gold (66ml) was slowly added to 25ml of freshly prepared conjugate according to the procedure of Horisberger and Rosset (1977). The mixture was continuously manually swirled for 2 minutes after addition. Six milliliters of 1% polyethyleneglycol (PEG) (MW 20,000) was added and the mixture was then 0.45u filtered. The mixture was then centrifuged at 11,500rpm at 4°C for 1 hour. The supernatant containing any unbound insulin, albumin, or glutaraldehyde was discarded. The GAI pellet was resuspended in 6ml of 0.1M phosphate buffer, pH 7.6, containing 4% polyvinylpyrrolidone (PBP) plus 0.2mg/ml PEG. 0.5mg/ml of sodium azide was added as a preservative. A total protein assay at O.D. 595 indicated that GAI had a concentration of 200ug/ml. The labell was diluted 1:4 for a working solution with buffer. Bovine serum albumin (BSA) was the reference standard.

GAI Histochemical Procedure

Cells were normally incubated with the GAI labell for up to 1 hour at room temperature. Controls consisted of cells or thin sections incubated with no labell, the insulin-bovine serum albumin conjugate, free insulin, free BSA, unlabelled gold, and free insulin followed by GAI.

Modified Lowicryl K4M Embedding Procedure

The following procedure was modified from the Polyscience, Inc. The tissue was minced into 0.5mm^3 pieces in 0°C fixative (PG or PGPA) and immediately transferred to fresh cold fixative at 0°C with two changes of fixative over a one hour period. The tissue was then transferred to 70% EtOH (-20°C) with two changes at 30 minute intervals. The tissue was then placed in 1:1 K4M:70% EtOH for 30 minutes (-20°C) then in 2:1 K4M:70% EtOH for 30 minutes (-20°C) then in 100% K4M for 30 minutes (-20°C). The tissue was then placed in 100% K4M overnight with initiator (complete K4M) (-20°C). The next morning the tissue was placed in fresh complete K4M for 60 minutes (-20°C) then placed in gelatin capsules with complete K4M and polymerized with UV light (-20°C) for 8 hours to overnight. The polymerized blocks were trimmed and sectioned. Thin sections were exposed to

labell for up to 1 hour, stained with 1% aqueous uranyl acetate and lead citrate, then observed under TEM.

CHAPTER IV

RESULTS

Ferritin-Insulin Marker

Ferritin-insulin (F-I), an electron microscopic (EM) marker prepared according to the method of Smith and Jarett (1981), demonstrated sparse binding on in vitro (whole-cell mount) and thin sectioned cells. F-I is considered a direct marker. Sparsity of labell on thin sectioned cells indicated evaluation of large surface areas, perhaps a different marker system. Cross sectional analysis of resin embedded cells shows approximately 1/100th of the total cell surface. With widely spaced receptors, either in groups or uniformly distributed, many sections may show little or no binding. Appropriate controls and antigen-antibody (Ag-Ab) binding tests along with comparative binding studies with cationic ferritin and ferritin-concanavilin A (Kincaid & Buthala, 1982) showed that F-I was an active and viable labell. In fact, the insulin radioimmunoassay (RIA) showed a 10 fold increase of insulin activity over that reported by Jarett and Smith (1977), Jarett, Schweitzer, & Smith, 1980).

These studies were primarily concerned with evaluating the efficacy of the F-I surface binding. Chinese hamster erythrocytes (Figure 4), primary human foreskin fibroblasts, human Type O erythrocytes, human Type O erythrocytes ghosts, and other tissues and cells of interest were exposed to cationized ferritin (Figure 5), ferritin-concanavalin A (con A) (Figure 6), or ferritin-insulin (Figures 8, 12, 15). The binding patterns were compared following thin sectioning (Figures 3-6, 13-15), cell surface replication (platinum shadowed carbon coated cell), (Figures 7-12), and scanning electron microscopy (SEM). Figures 4-6 show comparative bindings of cationic ferritin and ferritin-con A in comparison to the normal cell surface (Figure 3). Thin sections yield confident observations with dense labelling; however, sparse binding as reported for F-I required evaluation of large surface areas. The distribution of insulin receptors averaged $\sim 25/\mu^2$ mostly occurring in clustered patterns (Figure 12).

Gold-Insulin Marker

A colloidal gold-labelled insulin-bovine serum albumin (GAI) marker prepared according to the method of Ackerman and Wolken (1981) was used for ultrastructural visualization of cell surface insulin-insulin receptor binding sites. It is also considered a direct marker.

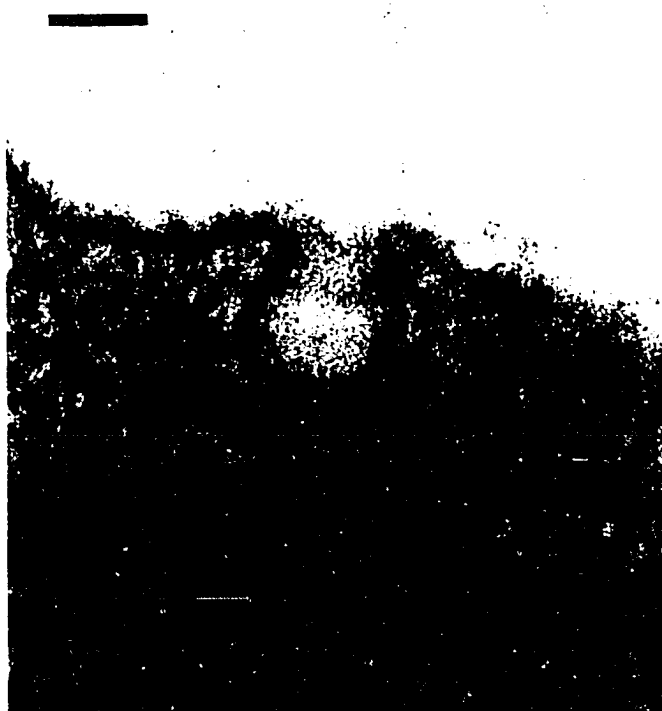


Figure 3. Normal Human Foreskin Fibroblast (HFF).
Bar = 0.1u.



Figure 4. Chinese Hamster Erythrocyte Labelled With
Cationic Ferritin.
Bar = 0.1u.



Figure 5. HFF Labelled With Cationic Ferritin.
Bar = 0.1u.

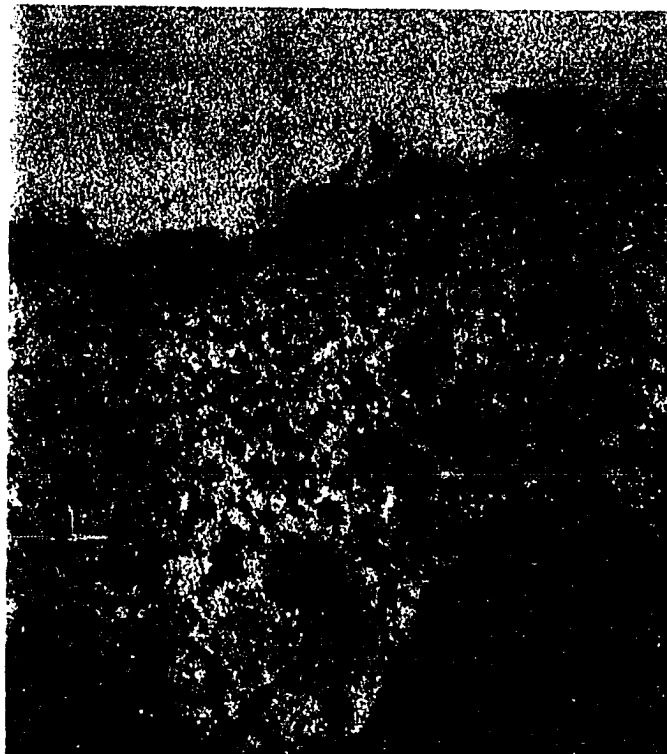


Figure 6. HFF Labelled With Ferritin Concanavalin A.
Bar = 0.1u.

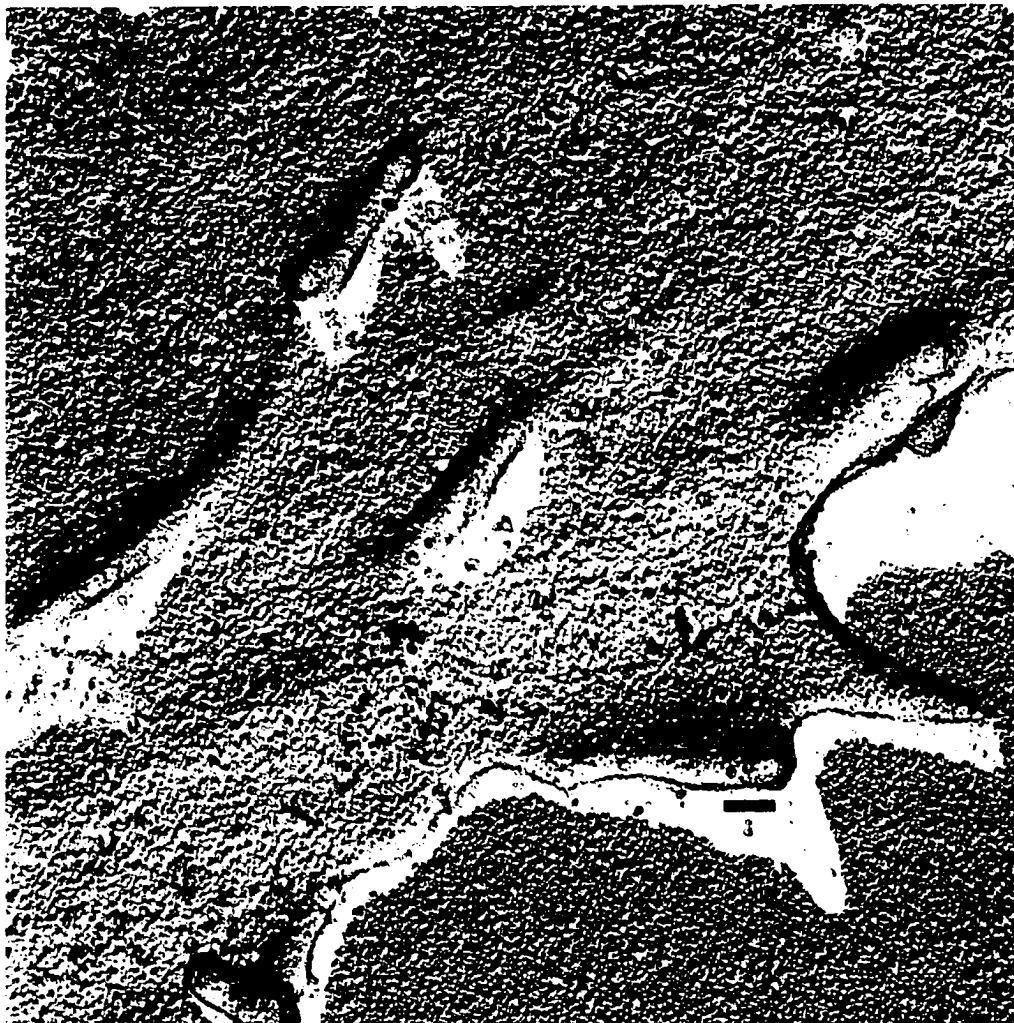


Figure 7. Platinum Shadowed Carbon Coated Replica of Normal HFF Grown on Glass Cover Slips.
Bar = 0.2u.

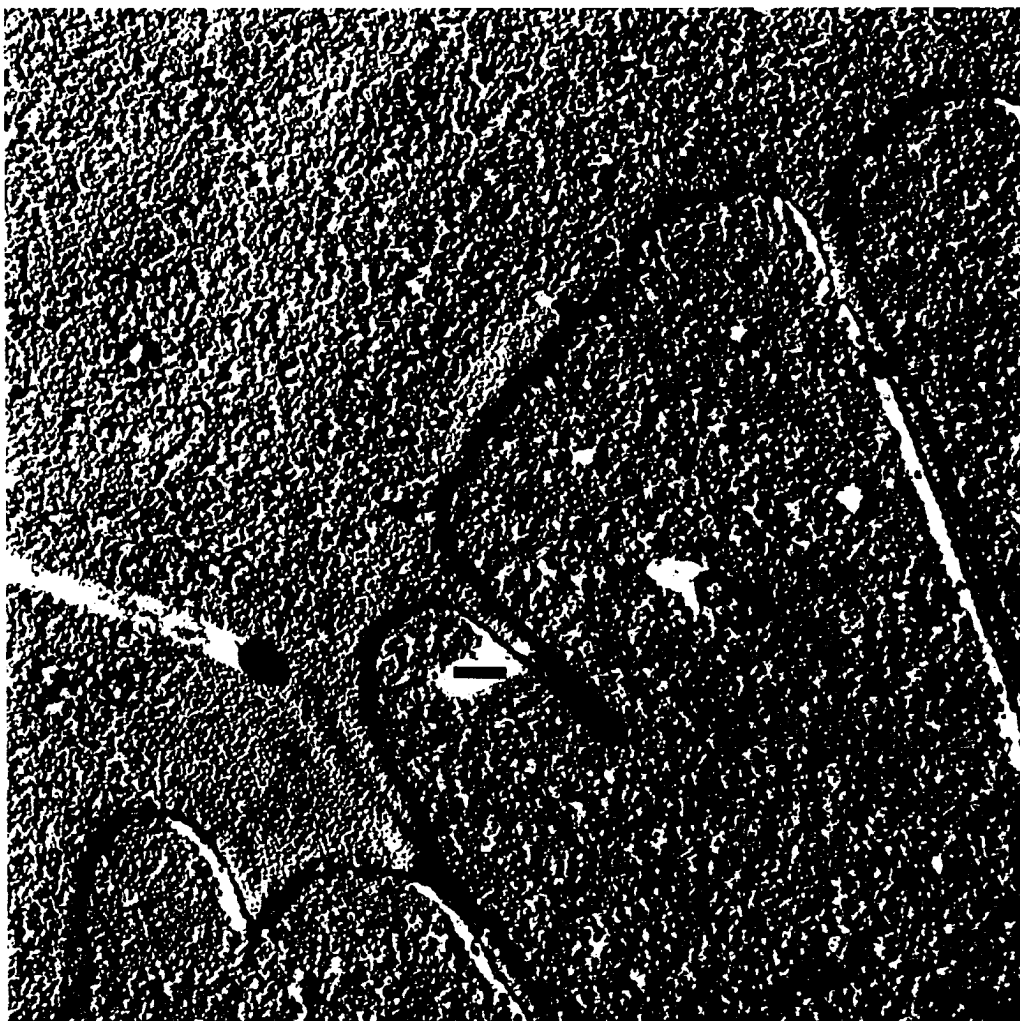


Figure 8. Replica of HFF Exposed to 5mU/ml Ferritin-Insulin (F-I) for 30 Minutes.
Bar = 0.2u.

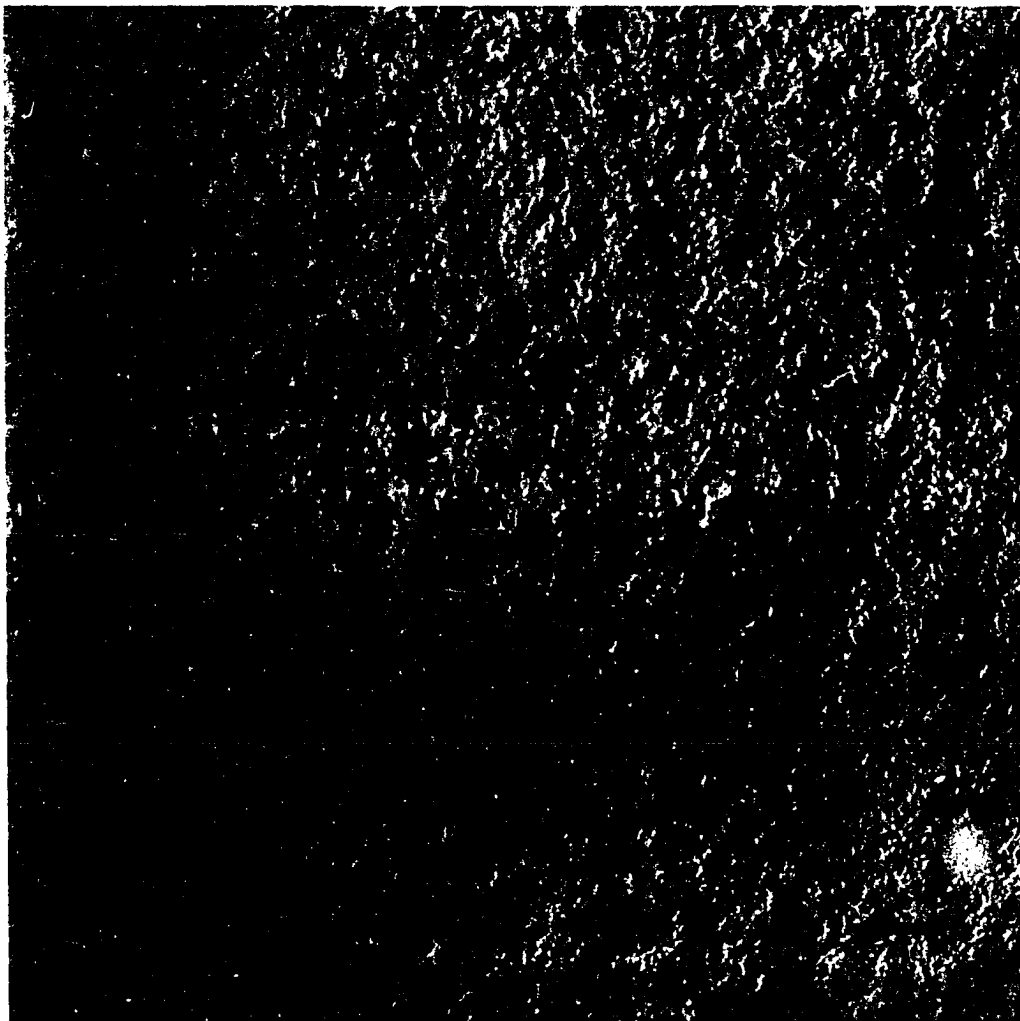


Figure 9. Replica of Normal Human Type O Erythrocyte (RBC).
Bar = 0.2u.

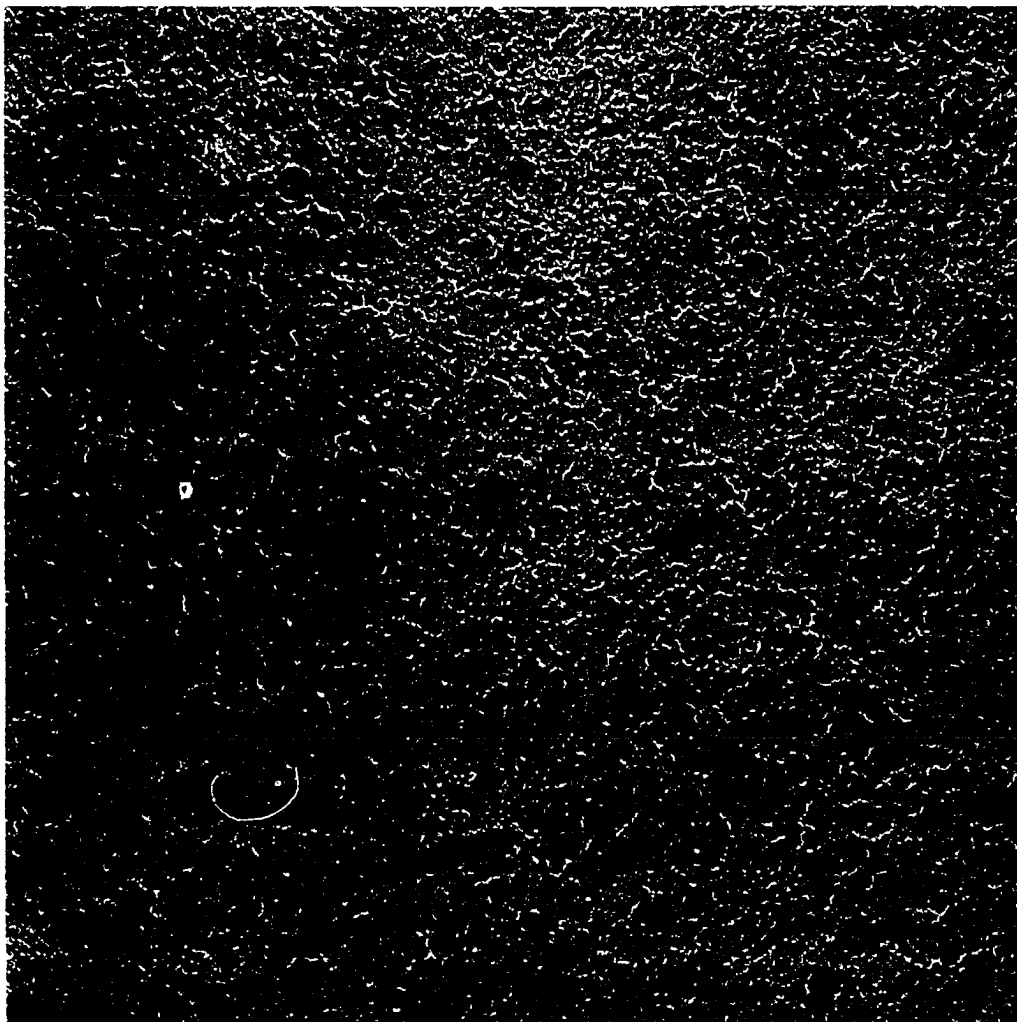


Figure 10. Replica of Normal Human Type O RBC Exposed to 120ug/ml Ferritin-Concanavilin A in 0.15N NaCl. Bar = 0.13u.

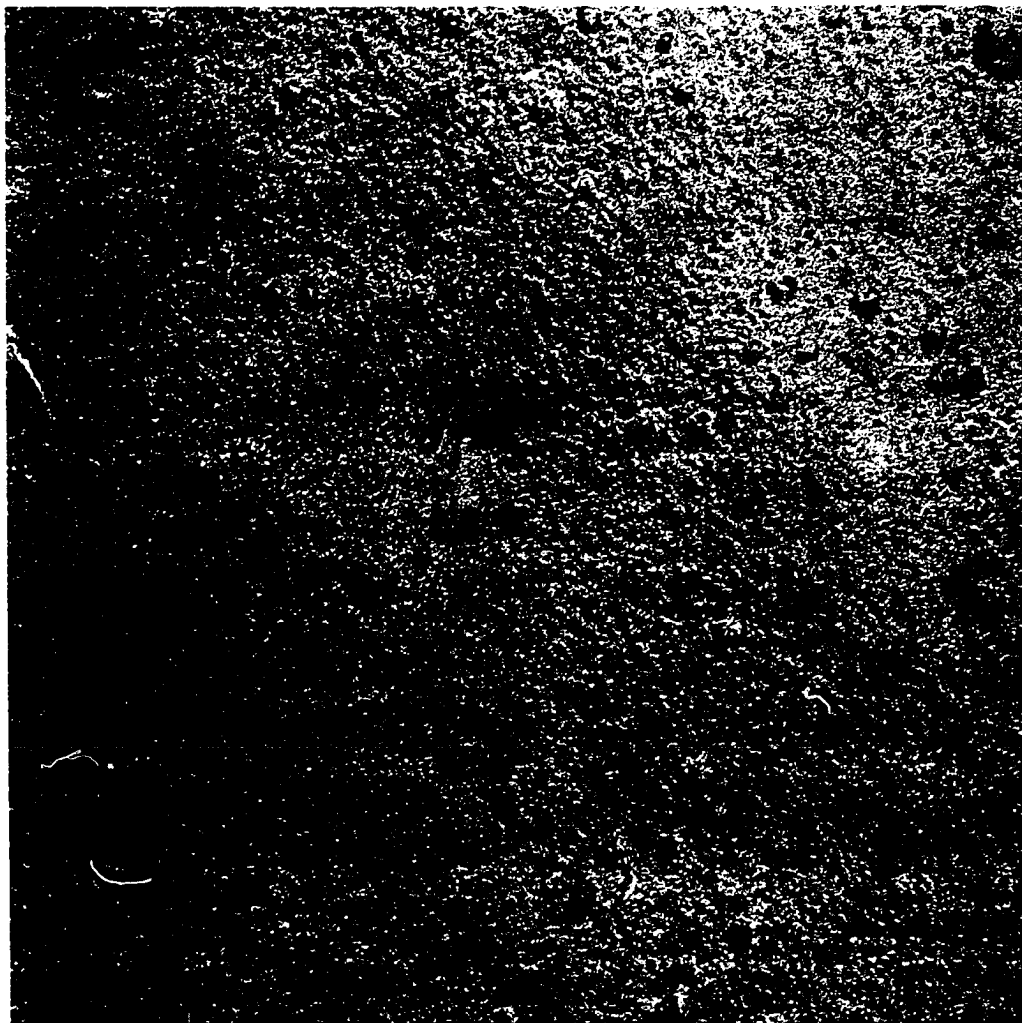


Figure 11. Replica of Normal Human Type O RBC Exposed to 120ug/ml Ferritin-Concanavilin A in 0.15 NaCl and 0.1% CrCl_3 .
Bar = 0.2u.

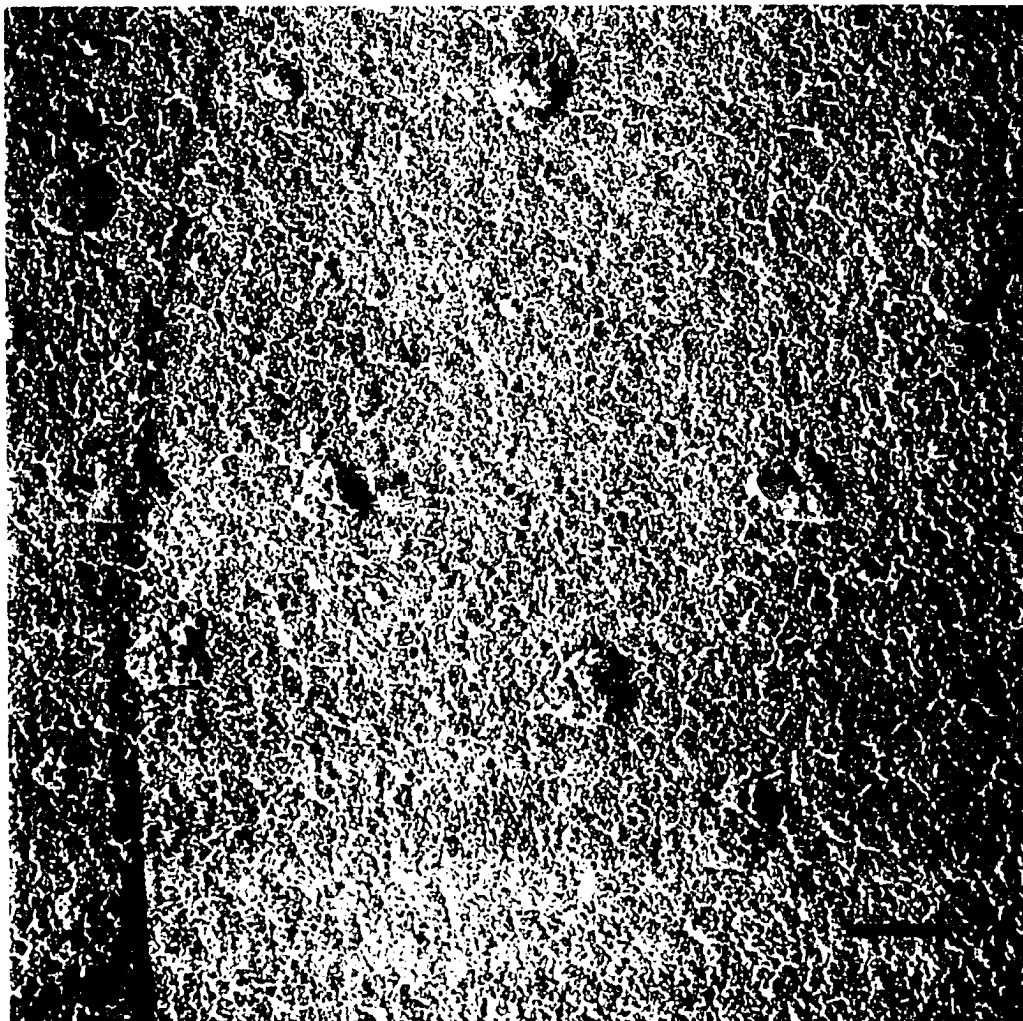


Figure 12. Replica of Normal Human Type O RBC Coated With Bovine Anti-Insulin IgG With CrCl_3 and Absorbed With F-I.
Bar = 0.1u.



Figure 13. Thin Section of Normal Human Type O RBC Embedded in Epon.
Bar = 0.13u.



Figure 14. Thin Section of Normal Human Type O RBC Embedded in Epon and Exposed to Ferritin-Concanavalin A After CrCl_3 Treatment.
Bar = 0.2 μ .

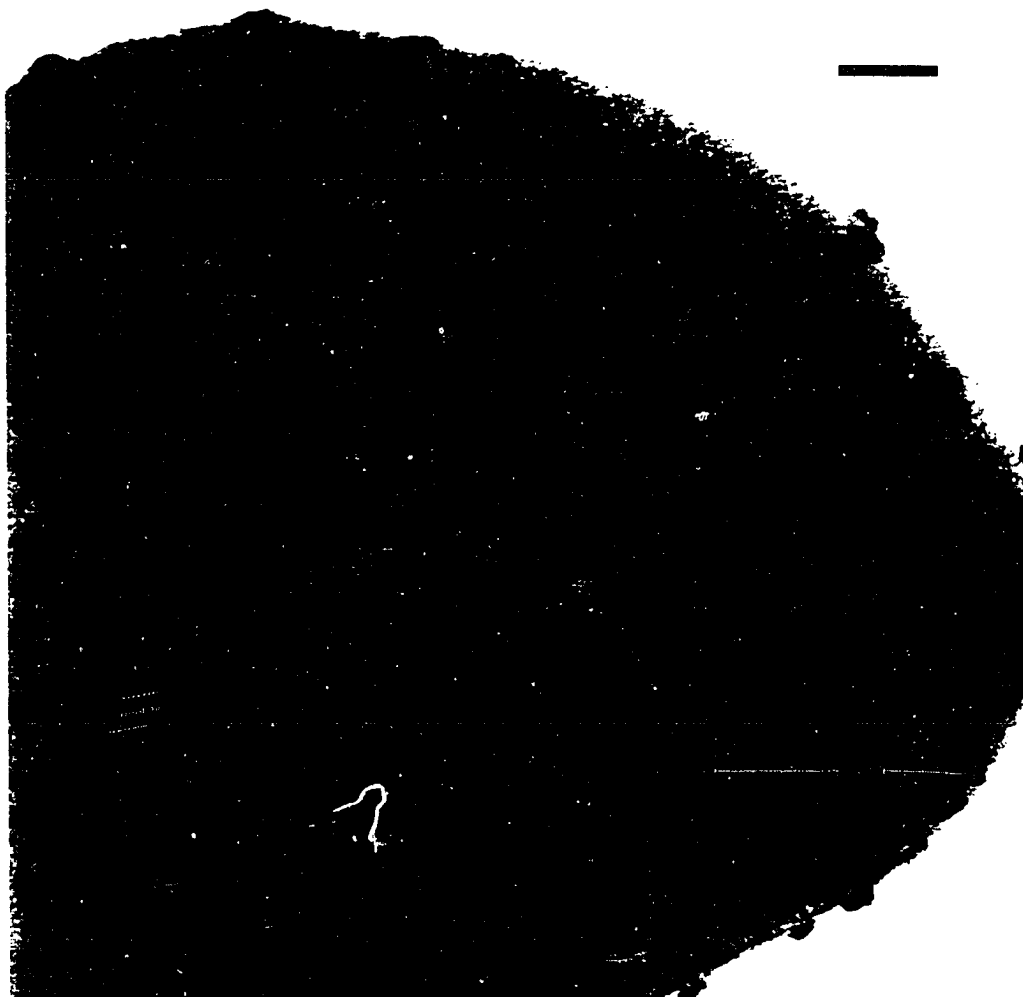


Figure 15. Thin Section of Normal Human Type O RBC Embedded in Epon and Coated With Bovine Anti-Insulin IgG With CrCl_3 Treatment Then Absorbed with F-I. Bar = 0.13u.

Again, sparse binding on in vitro cells was observed although not all comparisons were made as with F-I. Appropriate controls and Ag-Ab binding tests indicated that GAI was an active (functional insulin) and viable (visually demonstrated competitive binding) labell. Protein A-gold readily demonstrated its ability to localize anti-IgG immunoglobulin directed against pseudorabies virus (Figure 16) and normal mouse IgG (Figure 17).

Receptor Labelling Problems

In order to address some of the above problems with receptor labelling, additional studies were performed to alleviate fixation and embedding damage or masking of receptors. It was found for fixation that a combination of paraformaldehyde-glutaraldehyde (PG) or PG Picric Acid (PGPA) (Newman, Jasani, & Williams, 1983) worked best for EM than either aldehyde alone. In both fixative solutions the glutaraldehyde concentration was kept low (0.2%) to stop excessive crosslinkage of cell membrane components but high enough to preserve structural integrity i.e. a membrane stabilizing fixation.

Epon was best for ultrastructural preservation as compared to the hydrophilic methacrylate resins K4M and LR White. However, Epon an hydrophobic epoxy resin, employs many chemical agents for dehydration that are



Figure 16. HFF Grown on Formvar Film, Infected With Pseudorabies (PR) Virus, Absorbed With Porcine Anti-PR IgG and Labelled With Protein-A Gold (PA-Au).
Bar = 0.2u.

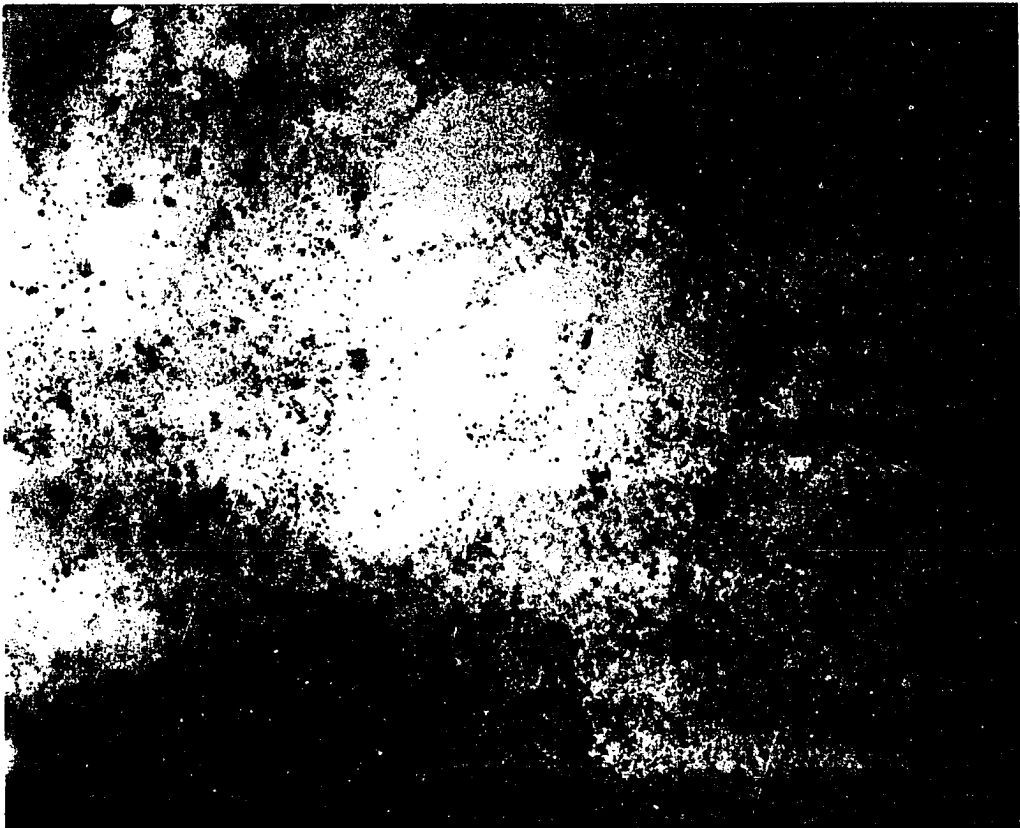


Figure 17. Normal Mouse Macrophage Grown on Formvar Film,
Absorbed With Goat Anti-Mouse IgG Then
Labelled With PA-Au.
Bar = 0.5u.

harsh on cellular structures and cause excessive molecular extraction. LR White gave the poorest ultrastructural preservation of cell structures with UV polymerization and even poorer preservation with peroxide polymerization with TEM. The latter was due to excessive heat generated during polymerization.

The best results occurred with PGPA or PG fixation and a modified embedding procedure for K4M that was carried out at deep freeze temperature. The tissues were passed from fixative directly to 70% EtOH then to the K4M resin for UV polymerization.

Cell Orientation

Cell orientation was achieved by in vitro growth of cell monolayers on ultra thin plastic film (Kincaid & Buthala, 1983). This type of procedure is generally referred to as whole-cell-mount specimens. The procedure allowed for quick and efficient processing for EM observation and avoided traumatic procedures involving enzymatic digestion and/or mechanical dislodgement. The film was either captured on 200 mesh EM grids or embedded for thin section study. The value of this procedure, in addition to ease of handling, was that cells remained naturally and intimately attached to the substrate undisturbed. Although not employed here, it also allows for stereoscopic analysis under TEM and 3-dimensional

analysis under SEM. With free unattached cells, there is a loss of cell orientation for the observer. Mechanical or other forms of cell removal cause damage and artifact and possibly loss of surface receptors. This procedure also restricts labelling of cells to one surface avoiding confusion of marker density. Since these thin plastic films range from 5 to 15nm thick, there was no difficulty for thin sectioning when embedded or for direct observation through the film when observed on EM grids (Figures 18-21). Macrophages are best suited for whole-cell-mount techniques since cell orientation is achieved as well as other criteria outlined in the discussion section.

Macrophages

Mouse and normal and diabetic Chinese hamster peritoneal macrophages grown on formvar film on 200 mesh stainless steel EM grids demonstrated ferritin and gold labels easily in cytoplasmic areas. Similar findings with whole-cell-mount methods have been reported by Takata and Hirano (1984) and Guagliard, Paulnock and Albrecht (1987). With lite (concentration and duration) PG fixation, ferritin markers tended to disrupt the cytoplasmic membrane. Higher magnification and/or marker intensification with bismuth poststain was necessary for visualization with ferritin labels but not with the gold



Figure 18. HFF Grown on Formvar Film and Labelled Unfixed
With Ferritin-Concanavalin A.
Bar = 0.2u.



Figure 19. HFF Grown on Formvar Film and Labelled Unfixed With Cationic Ferritin. "En Bloc" Stained With 1% UA in 70% Ethyl Alcohol. Bar = 0.2u.



Figure 20. Rabbit Kidney (RK) Grown on Formvar Film, Infected With Pseudorabies (PR) Virus, Absorbed With Porcine Anti-PR IgG, and Labelled With PA-Au. "En Bloc" Stained With 1% UA in 70% Ethyl Alcohol.
Bar = 0.2u.



Figure 21. HFF Grown on Formvar Film, Infected With PR Virus, Absorbed With Porcine Anti-PR IgG, and Labelled With PA-Au.
Bar = 0.2u.

probe. Also, mitochondrial regions with gold labelling were discernable but areas at or near the nucleus were difficult to study (Figures 22-23).



Figure 22. Normal Chinese Hamster Peritoneal Macrophage
Grown on Formvar Film.
Bar = 1.66u.

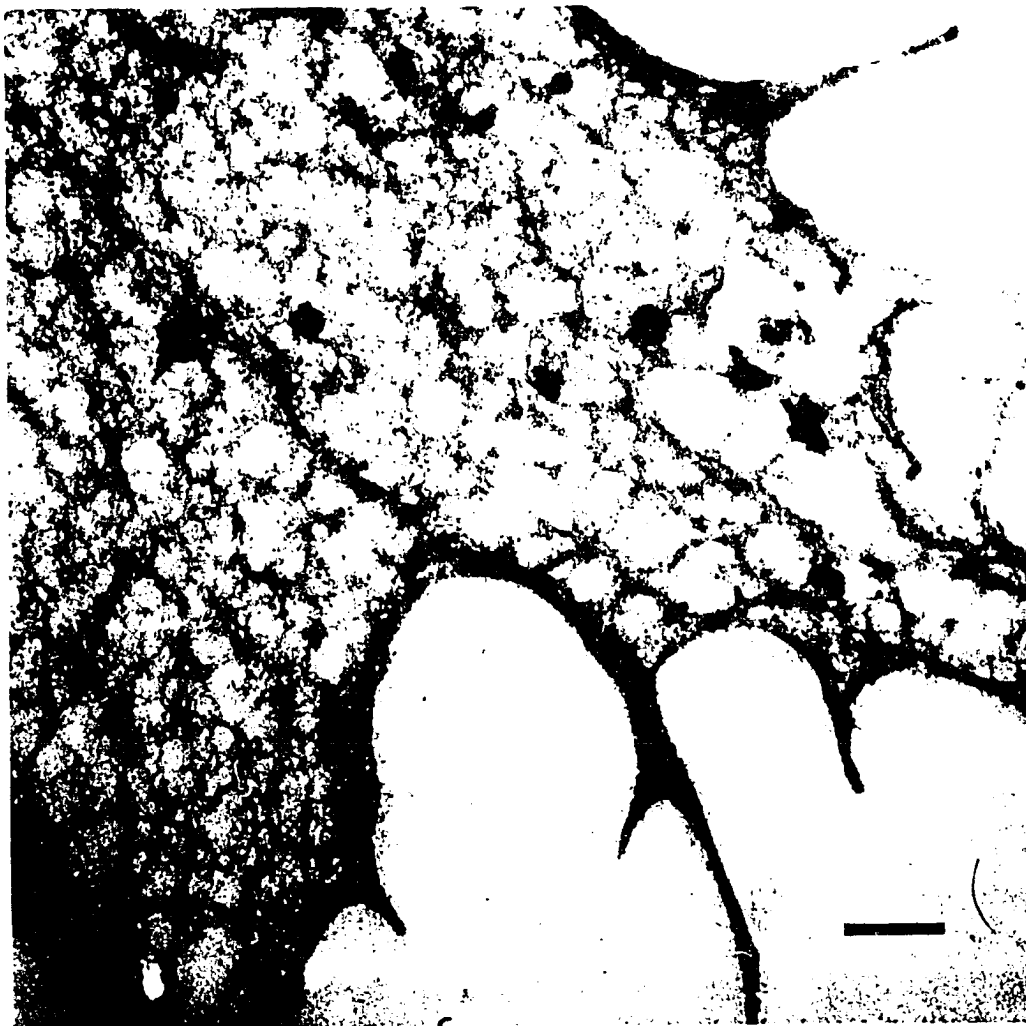


Figure 23. Normal Chinese Hamster Peritoneal Macrophage
Grown on Formvar Film.
Bar = 0.5u.

CHAPTER V

DISCUSSION

The introduction of particles bound to various molecules have facilitated studies of cell surface and intracellular tracing. One major problem is the inability to show surface binding of small bound particles. Even a cross section of an embedded thin sectioned sample may reveal no more than 1/100th of the total surface of the embedded cell. With widely spaced receptors many sections may show little or no binding. For this reason whole-cell-mount methods are preferred. In most all instances reported in the literature, ferritin conjugates show identical binding activity as unconjugated molecules. The same is true for gold adsorbed molecules. The reported distribution of insulin receptors is about $20/\mu^2$ (Smith & Jarett, 1988) occurring often in clustered patterns (Rosen, 1987; Smith & Jarett, 1988). Similarly we found both sparse F-I and GAI labelling on thin sections and whole-cell-mounts.

F-I does, however, have several drawbacks. One drawback is nonspecific binding which was very difficult to eliminate. A second drawback is steric hindrance of the covalently linked insulin to ferritin. A third

drawback is preparation, which is time consuming requiring separation and purification steps. A fourth, more critical problem, was visualization with TEM. Although ferritin is a fairly good electron emitter and fairly large in diameter (12nm), it blended too closely with cell structures and was difficult to observe in areas other than the cell surface, vesicles, and thin cytoplasmic membranes. However, with the more recent use of bismuth subnitrate post staining (Ainsworth & Karnovsky, 1972), the electron opacity of ferritin is vastly improved by absorption of this compound.

Labelling Comparison

To address the issue of sparse labelling, the procedure of Bendayan (1984) was followed to determine the presence of insulin instead of insulin receptors in normal mouse pancreatic tissue. However, this procedure was not performed in conjunction with F-I and GAI insulin receptor labelling studies. Similar tissue was used before for the labelling of insulin receptors with F-I and GAI. The tissue was embedded in Epon, sectioned and then labelled with goat anti-mouse insulin IgG/protein A-gold. The results with appropriate controls (Figure 24) were consistent with reported findings showing dense gold labelling in secretory (zymogen) granules (Figure 25). Etching of Epon thin sections with sodium metaperiodate

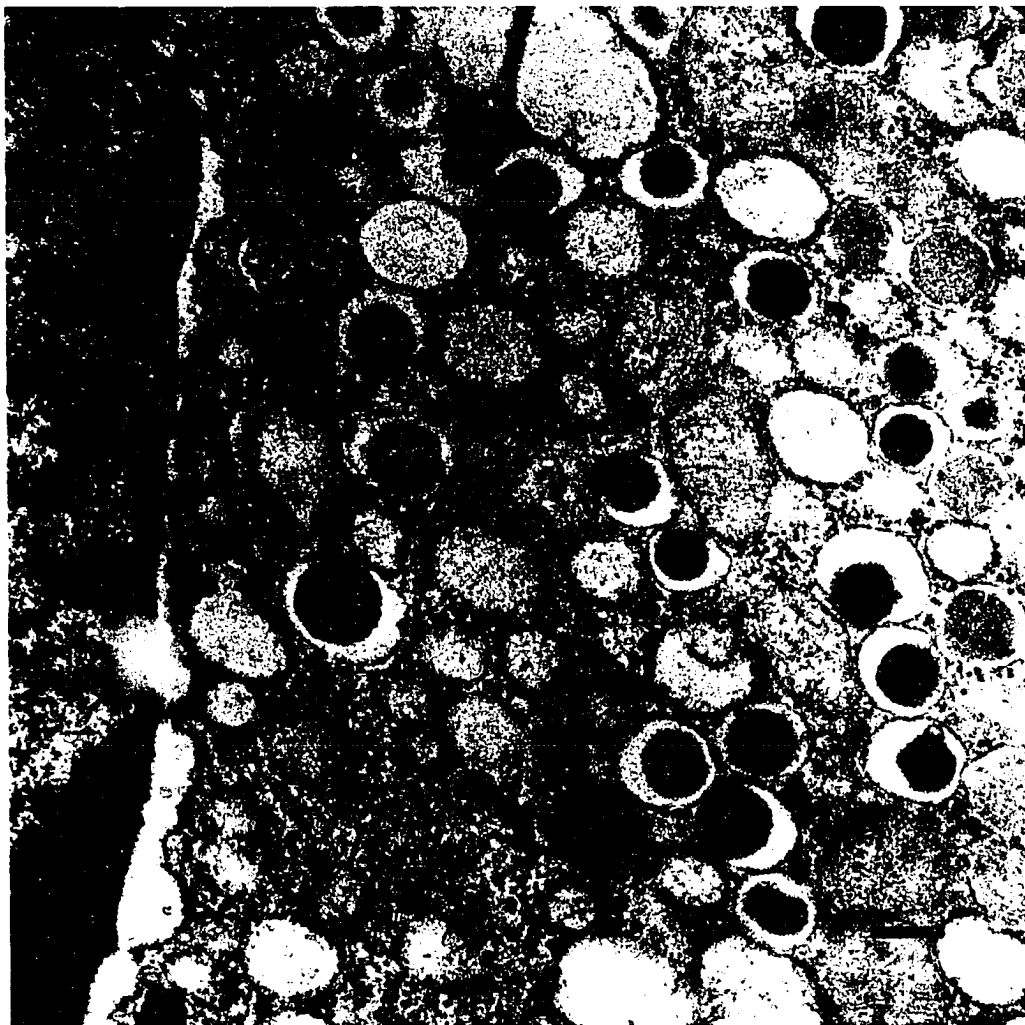


Figure 24. Thin Section of Normal Mouse Pancreatic
Tissue Embedded in Epon.
Bar = 0.26u.

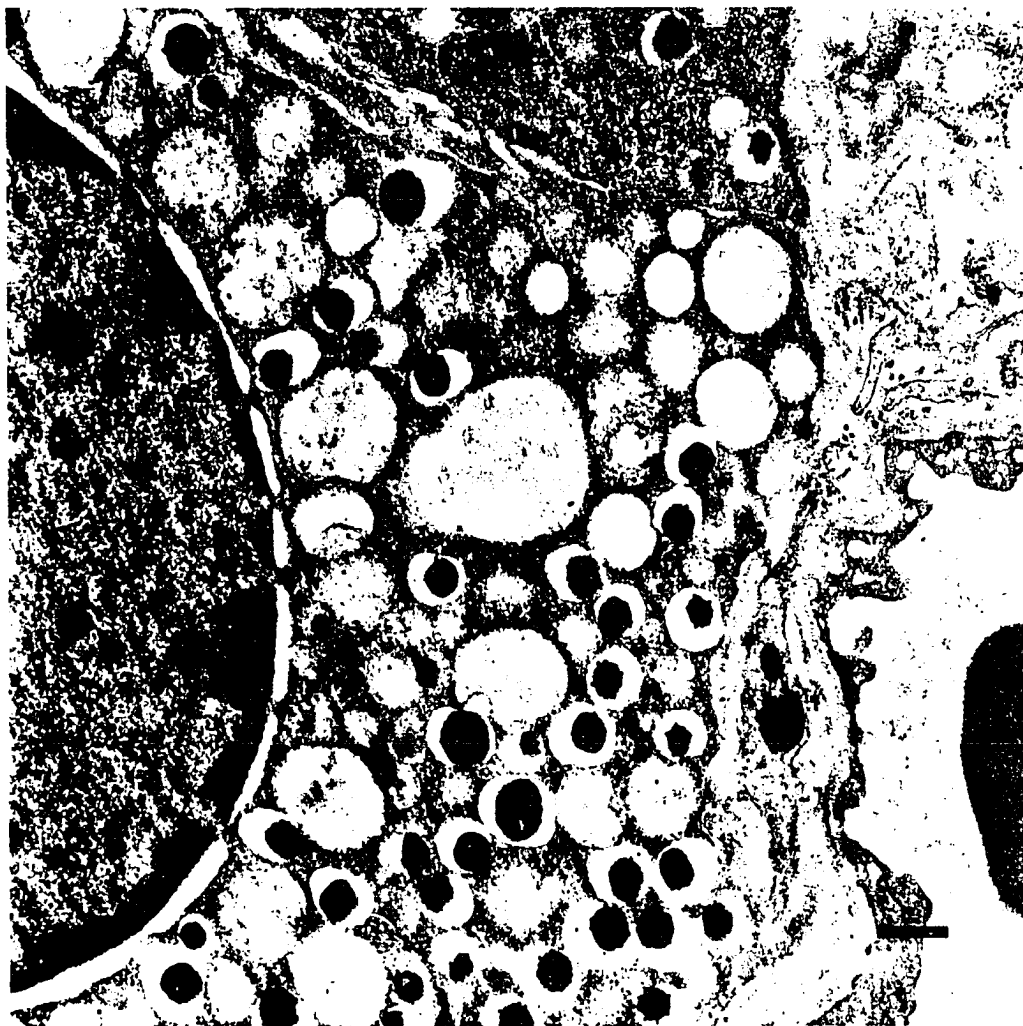


Figure 25. Thin Section of Normal Mouse Pancreatic Tissue Embedded in Epon, Absorbed With Goat Anti-Mouse Insulin IgG, Then Labelled With PA-Au.
Bar = 0.42u.

to unmasked insulin receptors sites was clearly not needed as some investigators claim.

When compared to F-I, the GAI was much easier to prepare and isolate. Also, potential damage to the insulin molecule during marker preparation was possibly reduced due to the fact that insulin is electrostatically adsorbed to the gold surface versus insulin be covalently linked with ferritin. In addition, the gold particles were slightly larger (17nm) and more electron dense than ferritin molecules (12nm) and could be more readily distinguished at relatively low magnification permitting the entire cell to be observed and photographed on a single plate thereby facilitating both visual and quantitative analysis of cell labelling. Steric hindrance resulting from the relatively large size of the labelled insulin complexes as compared to native insulin represents a potential disadvantage of both ferritin- and gold-labelled insulin used in cytohistochemical studies. With the GAI marker the albumin acts as a linked arm thereby reducing the steric effects on insulin caused by adsorption to the larger gold particle. Essentially the albumin is adsorbed to the gold surface with the linked insulin at the free unabsorbed end of the complex. Currently many forms of the gold labelled marker in various sizes are commercially available as indirect markers. This makes them more versatile for different

types of studies than direct more specific markers. The problem of sparse labelling for insulin receptors could have come from several potential sources:

1. Contamination of receptors by naturally existing free insulin or insulin introduced from growth medium.
2. Few insulin receptors exist for these cells.
3. Fixation could have damaged receptor sites by crosslinkage or mechanical damage.
4. Receptor masking due to embedding medium.

Etching of epoxy resin thin sections with sodium metaperiodate (Bendayan & Zollinger, 1983) or use of hydrophilic methacrylate resin (Roth et al., 1981) can reduce masking effects.

The ability to show surface binding of gold particles, without embedding and thin sectioning the sample, was not thoroughly tested with the thin film methodology.

Whole-cell-mount techniques offer a wide viewing surface for locating widely spaced labelled receptor molecules with EM. Other advantages with whole-cell-mount methods are: (a) these cells are readily accessible to researchers, (b) the cells remain 1^0 cells (as compared to altered transformed cells), (c) there is no removal from the substrate to which they are attached, (d) the cells attach and spread rapidly, (e) it is

relatively easy to establish a methodology for the use of whole-cell-mount cells.

These advantages are evident with peritoneal macrophages. First, the cells are readily accessible since they are easily removed from the peritoneal cavity after a cell lavage using heparinated growth media enriched with fetal calf serum and lavage. Second, the entire procedure is finished within several hours; no subculturing is required hence the cells remain relatively unaltered. Third, since transfer of cells is not required traumatic events of enzymatic treatment and/or mechanical dislodgement are avoided. Forth, the fact that the cells are hardy and tenacious allows the researcher to work with them shortly after culturing (planting). Fifth, the methodology requires that the cells remain intimately and naturally attached to an ultrathin plastic film. This affords the viewer with EM a sense of cell orientation and direction. When spread out the cells are thin thus allowing TEM penetration in cytoplasmic areas. Because the cells remain attached to the substrate, receptor labelling is confined to one surface of the cell. With free unattached cells in suspension, marker labelling occurs around the cell. This hampers efforts to estimate number frequency when using TEM to analyze distribution of surface markers since both surfaces are viewed as one. With attached

cells, background labelling is also confined to one surface thus avoiding the compounded confusion encumbered with the labelling of free cells.

Fixation

PG and PGPA gave better ultrastructural preservation for EM than either aldehyde alone. Paraformaldehyde penetrates tissue more rapidly than glutaraldehyde but only temporarily stabilizes structures that are more permanently fixed by glutaraldehyde. Both work well for crosslinking proteins; para- is faster and glutaraldehyde more permanent. Lipids, glycogen, and mitochondria are not preserved well by PG alone. Therefore, picric acid was used to prevent extraction during dehydration steps and preserve finer more delicate details of cell structure.

Controls

Controls are necessary in cell labelling studies in order to: (a) determine nonspecific binding of the labell, (b) show inhibition (blocking) of the labelled marker molecule by the unlabelled molecule, (c) compare normal unlabelled cells with labelled cells. These types of controls were used in all phases of this research.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

It is not conclusive from this research that there are receptor differences between diabetic and nondiabetic cells because of sparse labelling of receptors on the cell membranes. There is evidence by Ackerman and Wolken (1981) that glutaraldehyde fixation prior to label exposure prevented surface labelling of the insulin receptor. However, the presence of insulin was clearly demonstrated with these markers in normal tissue.

More research is required to understand the nature of F-I and GAI binding activity with their cellular receptors. However GAI is a valuable probe for investigation of receptor mediated processes in normal and diabetic cells. The gold methodology offers the following advantages:

1. Gold sols can be reproducibly, rapidly and easily prepared in a range of sizes (5 to 150nm in diameter), making the system extremely flexible (e.g. for multiple marking studies).

2. Gold probes are relatively inexpensive and easily prepared and purified, retain most of the binding activity of the unlabelled identifier or linker and are

stable for a long time.

3. Gold probes show little nonspecific adsorption.

4. Gold probes are electron dense easily detected by TEM, capable of strong emission of secondary and back-scattered electrons for SEM (no heavy metal coating needed). Gold emits characteristic x-ray signals which can be used to identify markers on cell surfaces.

5. Gold probes bound to cell surfaces can be quantified.

6. Gold probes are suitable for marking intracellular binding sites on thin sections.

7. Gold probes absorb or reflect light, and can be used also for subsequent silver precipitation. Thus, they are applicable for a variety of light microscopy (LM) and immunoblotting techniques.

Whole-cell-mount procedures are relatively simple, fast, and allow for conditions which mimic as closely as possible natural host conditions. For these reasons and evidence in the literature that most somatic cells (if not all) have receptors for protein hormones (Takahashi and Tavassoli, 1983) and perhaps for all circulating biomolecules, peritoneal macrophages from diabetic and nondiabetic Chinese hamsters with gold labelling techniques would be ideal for continued focus on insulin receptor research with electron microscopy.

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