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LETTER TO THE EDITOR

HUMAN AFFERENT LYMPH CONTAINS APOPTOTIC CELLS AND "FREE" APOPTOTIC DNA FRAGMENTS – CAN DNA BE REUTILIZED BY THE LYMPH NODE CELLS?

In his article entitled "High Points in the History of Lymphology 1602-2001" (1), JW Shields cites the historical works on the *in vivo* and *in vitro* observations of lymph cell fragmentation (clasmatosis) into tiny amorphous fragments. This process proceeds without nuclear changes; that should differentiate it from apoptosis. The shed, as he cites "hyaline bodies," are soluble globulins. He also writes, based on articles of Fichtelius, Craddock, Bryant and Dumont from the 1960s and his own suggestions from the 1990s that "lymphocytes donate DNA to enhance growth of neonatal gut and lymph glands, restore integrity of liver cells, enhance capacity of fibroblasts to heal wounds". Continuing the problems of DNA, he states that concentrations of DNA in lymphocytes are 20x greater than in other cells and these cells should facilitate the transport of DNA to other tissues. The citations from old works, although of basic historical value, should be viewed with extreme caution to avoid drawing wrongful conclusions. Not all of them have proved to be accurate when re-analyzed with modern research techniques. With even more caution one should look upon the suggestions of Shields (2,3) that lymphocytes "donate" to other cells, shed cytoplasmic globulins and nuclear DNA. This vision, although extremely attractive, properly should remain in the realm of hypothesis until objective evidence is provided. Our group has been working on the problem of reutilization of cellular debris for some time. It was in 1977 that we demonstrated that human leg afferent lymph, collected from a cannulated superficial lymphatic, contains cellular debris not seen in whole blood samples (4). Fragments of membranes, nuclei, mitochondria and fibrinogen were seen on electron micrographs. These pictures pointed to *in vivo* necrosis or apoptosis of lymphoid cells rather than shedding of cytoplasmic bodies, although cytoplasmic membrane protrusions were quite dense. Moreover, the concentration of antibody-producing B cells remained below 2% (5) and the concentration of various subclasses of antibodies reached only 10-25% of that of serum (6), and was similar to the concentration of other proteins filtered from plasma. Thus, no local source of antibodies could be documented in the whole lymph samples. Observations made during *in vivo* cultures of lymph or blood cells with PHA for periods of 1-3 days are irrelevant, because approximately 50% of cultured cells undergo necrosis due to artificial culture conditions and cellular debris lines the bottom of culture wells. Other interesting observations were made by us studying the *in vivo* collected lymph. Up to 20% of leg afferent lymph lymphocytes, but not dendritic cells, underwent apoptosis. The local factors responsible for this high level of nuclear disintegration remain unknown. The nuclear DNA underwent fragmentation to 400bp and below, whereas the cell membrane remained intact. A typical "ladder" structure of DNA

was seen on the PCR lanes (8). The apoptotic process is regulated by the Fas-FasL (ligand) interaction and has initially been called "cell suicide." The apoptotic cells or their nuclei are ingested by dendritic cells, sometimes also by macrophages. Then, the ingested or freely floating apoptotic cells are transported with lymph to the nodes where the interdigitating dendritic cells process the nuclear material and present it to lymphocytes. Cohorts of primed lymphocytes are raised. This process is called cross-priming and results in induction of tolerance to self-antigens contained in the apoptotic cells (7). On the other hand, if the apoptotic cell contains bacterial DNA or viral particles, an immune process develops with clinical symptoms of inflammation. We have found that normal human leg afferent lymph contains free fragments of apoptotic DNA (7). The concentration of "free" fragments of DNA in lymph from patients with rheumatoid arthritis or lymphedema are higher than that of normal subjects. The distribution of fragments of different numbers of base pairs differed between lymph and serum. The question as to whether the DNA from the apoptotic bodies or "free" DNA fragments can be reutilized by the lymph node or cells of other origin has so far not been verified. The process of "reutilization" of DNA fragments needs favorable conditions for penetration of the cellular and nuclear membranes, de-spiraling of recipient DNA, "fitting" of the donor fragment to the "free space" in the recipient DNA strand and other conditions necessary for recombination. On the other hand, is the fragmented DNA only a waste product of enzyme disintegration? It seems highly unlikely. Successful gene transfer and therapy has brought about reversal of various metabolic disorders. Direct transfer of DNA to the skin currently represents a potential therapeutic approach to the systemic delivery of active molecules (9). However, before we can legitimately proclaim that "DNA can be donated by a cell to another cell," clear scientific documentation needs to be provided for that putative process.

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Reply:

Olszewski's studies, as referenced (3-8) are limited to human subcutaneous peripheral lymph. My studies have been on organized lymphomyeloid tissues, including the adenoids, tonsils, spleen, thymus, lymph nodes, gut associated lymphoid tissues (GALT), and bone marrow of humans, rats,

pigeons, chickens, humming birds, water fowl, submerging turtles, frogs, goldfish and mosquito fish with a focus on the central circulating lymph and blood cells which these tissues and the cloacal bursae of birds produce. The methods of study have been via standard hematologic smear and thin tissue sections, supplemented with transmission and scanning electron microscopy sequentially at the Mayo Clinic, University of Illinois, Santa Barbara Medical Clinic, Santa Barbara Cottage Hospital, University of California in Santa Barbara, and for a short, but productive period in 1980 with Arnfinn Engeset in Oslo. My formal training is in internal medicine, hematology and clinical pathology. Animal studies were performed with professional biologists or ornithologists. We have not studied identical material, and it's not certain which methods are best, and what conclusions will ultimately prevail.

The process of clasmatosis from mononuclear cells, including reticulum cells, macrophages, large lymphocytes, medium-sized lymphocytes, plasmacytes, and megakaryocytes is well documented by many including my own studies via smear technology, transmission and scanning electron microscopy (TEM and SEM). The process is difficult to demonstrate in formalin-fixed tissue sections, because the fixative produces 30% shrinkage of tissues owing to dehydration. However, glutaraldehyde, as a fixative, greatly reduces this problem, especially when used for TEM and SEM. Whereas it might seem obvious to many hematologists that lymphocytes normally reduce in size and increase in nucleocytoplasmic ratio via clasmatosis during differentiation in the organized lymphoid tissues of all animals, the process is still not widely recognized, along with the gel-sol relationships which obtain between cytoplasm, interstitium and circulating lymph. Instead of clasmatosis, the terms cytoplasmic blebbing or shedding have been used more recently. Usually, when mononuclear cells bleb, shed or otherwise exhibit fragmentation, the ectoplasm extrudes in the form of plasmalemma-encased globules wherein the mitochondrial, ribosomal and amorphous contents swell from water imbibition and become hyaline with dispersion until the plasmelemma ruptures and the visible structures in the cytosols disappear into the interstices and central lymph. Bits and pieces of polyribosomes, ribosomes and mitochondria are usually visible in the extruding globules, as well as in the interstitial ground substance and central lymphatics. However, few, if any are visualized in arterial or venous blood after the central lymph has circulated through the lungs, heart and capillary circulation.

With respect to the small cytoplasm-depleted lymphocytes (SCDL)which normally emanate from organized lymphoid tissues of all animals ad infinitum after embryonic gastrulation, such cells are eukaryotic and probably contain no more or less DNA than other kinds of cells. However, because of nuclear condensation and loss of cytoplasm during maturation, they contain concentrations of DNA 5-10x greater than that of most remaining body cells. The extreme differences (e.g., 20x) are from comparing the SCDL with neurons whose nuclei are small compared with their extensive and dendritic cytoplasm. Being small and compact in size, as well as emperipoletic within interstices, between other cells and into other cells, the SCDL are eminently capable of carrying relatively great concentrations of DNA into other tissues, as well other cells, especially just prior to mitosis when their own DNA is unstable and likely to incorporate exogenous DNA. Fichtelius, Craddock, Bryant and Dumont showed with isotopic labeling of lymphocyte DNA that relatively large quantities are reutilized in the cells of the neonatal gut, lymph nodes, fibroblasts in healing wounds and in regenerating hepatocytes. To date their observations and conclusions, along with my own remain to be disproved, as well as more widely broadcast.

With respect to apoptosis (falling apart)

vs clasmatosis (fragmentation), neither is a perfect term. Currently, apoptosis connotes sudden mitochondrial caspase-induced nuclear and cytoplasmic disintegration. The term, clasmatosis, describing progressive cytoplasmic fragmentation is seldom used nowadays. Apoptosis further connotes cell death or suicide, while clasmatosis describes a form of exocytic secretion observed in large lymphocytes and plasmacytes during maturation and in macrophages after ingestion and digestion of particulate matter. Apoptosis in SCDL is commonly observed in blood smears, in intraepithelial SCDL and in organized lymphoid tissues (especially thymus) during stress or adrenal glucocorticoid stimulation. The term, lymphocytolysis, was used formerly to describe this phenomenon, possibly attributable to mechanical or osmotic fragility, or cortisol-induced changes leading to rapid release of normal and immune globulins into the blood circulation (1). In tissue culture, emperipoletic SCDL have been observed to induce apoptosis in co-cultured epithelial cells on surface contact, to undergo apoptosis inside, or wander through without apparent interaction (2). Apoptosis, also called programmed cell death, is now considered common in many different kinds of cells in many species of vertebrates during various stages of development as a means for riddance of senile, effete or useless cells during embryogenesis and adult life (3). Apoptosis seems to be the outcome when sensitized lymphocytes reject genetically incompatible cells (3). Then, the bottom line is: "What happens to the remains of each apoptotic cell in accordance with standard concepts of mass/energy, as well as water conservation in humans, as well as in other animals?"

From a histologic point of view, the bulk of clasmatosis with cytoplasmic fragmentation in human lymphoid tissues occurs from large and medium sized lymphocytes, plasmacytes and macrophages supported by reticular ground substance. The bulk of apoptosis with nuclear and cytoplasmic degeneration usually occurs within large germinal center macrophages (LGCM), in periarteriolar dark lymphocytopoietic poles of secondary germinal centers, in thymic epithelial reticular cells, and beneath the nucleoli of columnar epithelial cells or in the basal germinative layer of all stratified epithelia. The numbers of SCDL undergoing nuclear and cytoplasmic fragmentation appear proportional to the turnover rates of cells in each region (4). In the secondary germinal centers, the macrophages loaded with ingested SCDL nuclei in sundry stages of lysis appear to secrete myriad cytoplasmic globules into the ground substance formed by follicular dendritic cells and immediately adjacent to large germinal center lymphocytes also shedding globules, as they divide and transform into smaller lymphocytes with less cytoplasm with repeated mitotic divisions and continued cytoplasmic shedding. One may presume, therefore, that the bulk of SCDL DNA which is reutilized in lymphocytopoiesis is first processed, along with other ingested macromolecules in the LGCM. Conversely. in the periphery, the bulk of substances which arise through SCDL nuclear and cytoplasmic fragmentation appear to be reutilized more or less directly by the basal epithelial cells. Although follicular dendritic cells (FDCs) in the organized lymphoid tissues supply fibrils and ground substance through which macromolecular exchanges take place, they do not appear to play an active role in the digestive or secretory processes involved. In the periphery, the Langerhan "veiled" or dendritic cells do not appear to play an active role in the transport of emperipoletic SCDL or their apoptotic remnants to growing epithelial cells. However, what comes back from epithelia, especially the stratified squamous epithelium protecting the external surface of the body is another unanswered question.

Olszewski's careful studies on the contents of peripheral lymphatics, Langerhan cells and tissue macrophages add important

data on the circulation and recirculation of lymphocytes and their apoptotic remnants, as well as soluble DNA. Undoubtedly, his observations will find their proper place with time in the feedback regulation of the lymphatic apparatus, as defined by Drinker, Yoffey and Courtice and characterized by Claude Bernard (5) from a comprehensive point of view.

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