SUMMARY  Human atheromatous material was injected into the cerebral vasculature of anaesthetized rabbits via the left common carotid artery. The lethality of varying dosages was determined and the distribution and general character of occlusive vascular lesions which developed were analyzed by light and transmission electron microscopy. It was found that a dose exceeding 55 mg of the atheromatous material (125 mg/ml saline) was lethal in New Zealand white male rabbits weighing between 3 and 5 kg. In nonsurviving animals, parts of the Circle of Willis and usually one or more of its major tributaries were occluded. Some surviving animals exhibited signs of neurologic deficit evidenced by motor dysfunction. Occlusive vascular lesions found in surviving animals were predominantly localized in ipsilateral cortical and subcortical vessels within the distribution territory of the middle cerebral artery. The character of occlusive lesions showed strong evidence of thrombosis. These results demonstrated that this experimental system may be useful as a model for the study of blood-atheroembolic vascular reactions, cerebral infarction development and the testing of agents potentially prophylactic against the development or stabilization of occlusive lesions.

Cerebral Atheroembolism
An Animal Model

B. J. JEYNES, PH.D. AND B. A. WARREN, M.B., D.PHIL.

ATHEROEMBOLISM is increasingly recognized as a significant etiological factor in the development of neurological dysfunctions such as TIA, amaurosis fugax, and certain kinds of stroke.1-3 The frequency of the contribution of atheroembolism, contrasted with thromboembolism, to the incidents of these vasculopathies has most frequently been estimated to be between 10 and 33%.2-4 The primary source of cerebral atheroembolic material is believed to be the carotid bifurcation.9-12 and in general the area most often affected is the distribution territory of the middle cerebral artery.13, 14 The most frequently reported lesions are described as being multifocal and within the cortical and subcortical vessels, with only rare reports of these lesions in brain stem structures. The size of target vessels which become occluded are variously reported between 8 and 200μ,13, 15, 16 although 100μ vessels appear to be most frequently observed.17 The morphological character of the occlusive lesions is only scantily described in the clinical literature, particularly as this relates to acute lesions. However, the most characteristic observation is the presence of cholesterol crystals. The presence of thrombus material has also been noted both in the clinical literature18, 19 and in some of the experimental data18, 20-23. Current prophylactic and medical interventions against this phenomenon, such as the use of anticoagulants, are far less successful than desired. Thus it would seem desirable to establish an animal model in which sublethal doses of human atheromatous material (H.A.M.) could mimic the above general characteristics in order to come to a clearer understanding of the basic aspects of the pathogenesis of atheroembolic cerebrovascular occlusions. In this study we wanted to determine what the lethal dose of atheroma was in the selected animal model, in order to be able to reliably reproduce various kinds of acute and chronic lesions; and what the light and transmission electron microscopic characteristics of the lesions were; in order to compare them with clinical descriptions and observe what reactions might be occurring at the lesion sites. We have selected the rabbit as a suitable model for a number of reasons. The major vascular distribution is similar to that in man, except that there is a single anterior cerebral trunk and the superior cerebellar arteries arise from the Circle of Willis. The rabbit does not have any rete mirabile24-26 and thus the source of cerebral emboli is restricted and predictable. Finally, much of preceding atheroembolic research has been carried out in the rabbit,18, 20-23, 27-33 and thus useful comparative information is available.

The embolic quanta which experimenters have used to reproduce atheroembolism have consisted of either cholesterol crystals or human atheromata. Those who have injected cholesterol crystals alone have generally argued that human material in an animal model may provoke antigenic reactions which would compromise an accurate interpretation of the results. However, the morphology of the lesions produced experimentally compares closely with what few acute human lesions are described.34, 35 Components of atheroma have been observed to cause different reactions. There is strong evidence that cholesterol crystals can create obstructions within appropriately sized arteries but are relatively nonreactive, and if small enough are removed from the lesion site by macrophages.32, 33 There is also evidence that there are components of atheroma which are thrombogenic and/or inflammatory.23, 26 These latter sequelae may contribute more to morbidity and death, in that they may augment and stabilize an embolic mass which may otherwise have been pathologically benign. While it is certainly necessary to examine the blood and vessel wall reactions to the separated components of atheroma, it is equally necessary, for a
meaningful comparison with the synergistic realities in the human condition, to understand the provocative characteristics of whole atheromatous material.

Methods
Preparation of Embolic Material
Atheromatous gruel was removed from mature atherosclerotic plaques of human aortas taken at autopsy.22 The plaques were opened under surgically clean conditions and the gruel removed taking care to avoid removing any of the fibrous cap or scraping the wall or floor of the lesion. The material was then suspended in sterile saline at 125 mg per ml and ground in a manual tissue homogenizer. This suspension was then drawn up into a tuberculin syringe through a 27½ guage needle. Doses of between 30 and 80 mg were prepared.

Control dosages of 1 cc of saline and of 80 mg homogenated rabbit liver (125 mg per ml) were also prepared.

Animal Procedures
Thirty-eight male New Zealand white rabbits weighing between 3 and 5 kg were used in these experiments. The dose distribution was as follows:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (1cc)</td>
<td>4</td>
</tr>
<tr>
<td>Homogenated Liver (80 mg)</td>
<td>4</td>
</tr>
<tr>
<td>Atheromatous Material:</td>
<td></td>
</tr>
<tr>
<td>30 mg</td>
<td>3</td>
</tr>
<tr>
<td>40 mg</td>
<td>4</td>
</tr>
<tr>
<td>50 mg</td>
<td>5</td>
</tr>
<tr>
<td>55 mg</td>
<td>5</td>
</tr>
<tr>
<td>60 mg</td>
<td>4</td>
</tr>
<tr>
<td>75 mg</td>
<td>6</td>
</tr>
<tr>
<td>80 mg</td>
<td>3</td>
</tr>
</tbody>
</table>

Each animal received a single injection.

All injections were made into the surgically exposed left common carotid artery under Pentobarbital Sodium (Abbott Laboratories Ltd., Montreal) anaesthesia (approximately 15 mg/kg bw). Maintenance anaesthesia was by ether inhalation. The materials were introduced into the artery at a slow and even rate.

The puncture wound was closed by applying gentle pressure over the site for 2–4 minutes. Animals which survived the injection were sacrificed at times ranging from 10 minutes to several days, post injection. The behavioral characteristics of survivors were noted.

The brains of all animals were fixed under anaesthesia by standard cardiac perfusion procedures, with a perfusion pressure of 100 mm Hg, with either 3% phosphate buffered glutaraldehyde (for T.E.M. preparations) or 3% Sorensen buffered paraformaldehyde (for light microscopic preparations).

The brains were removed from the cranial vault and their exterior surfaces examined first. This included inspection of the blood vessels comprising the Circle of Willis and its tributaries. Gross inspection also included observation for evidence of surface encephalomalacia. The brains were then placed in a plexiglass cutting cradle and cut antero-posteriorly in 3 mm slices and displayed serially. They were then inspected for signs of infarction.

Whole brains and brain slices with exceptional features were photographed. This is a general adaption of the neuropathological method for examination of post mortem brains described by Kaufmann.37

Brain slices and random mm³ samples of tissue were processed for light and transmission-electron microscopy respectively.

Tissue Processing
For Light Microscopy: Brains for paraffin embedding and sectioning were placed into 3% Sorensen buffered paraformaldehyde after perfusion. They were examined and sliced as usual and then the slices were embedded in paraffin, sectioned (3–5 μ sections), placed on slides and stained with haematoxylin and eosin.

Light microscopy of cerebral tissue was also observed on Spurr embedded thick sections stained with 1% toluidine blue in a 1% borax solution.

For Electron Microscopy: After small samples were removed from the brain, they were cut to appropriately smaller sizes for preparation (approximately 1 mm cubes). These were then permitted to immersion fix for 2 hours in 3% phosphate buffered glutaraldehyde. The remaining process consisted of a sucrose wash, post fixation in 1% phosphate buffered OsO₄, dehydration in a graded series of alcohols, en bloc staining with 0.5% uranyl acetate and saturated lead citrate, Spurr embedding, polymerization and sectioning on an LKB Ultramicrotome III. Sections were observed on a Philips 300 transmission electron microscope operated at 60 KV.

Results
Animals which received 1 cc of saline or 80 mg of homogenated rabbit liver survived, without symptoms, until killed up to several days post injection. At autopsy no cerebrovascular lesions or infarcted parenchyma were observed. With one exception all animals given 55 mg or less of human atheromatous material survived. The non-survivor of this group received a dose of 50 mg. All animals given 60 mg or more of human atheromatous material died within seven hours.

However, three quarters of the animals died within two minutes of the injection. The animals which survived beyond the initial few minutes all showed signs of respiratory distress.

An animal given 30 mg and an animal given 60 mg, which survived 7 hours, both exhibited right to left head drift, a general inability to posture normally, some loss of muscle tonus, and a preference for a right sided resting posture.

In non-surviving animals portions of the Circle of Willis and ipsilateral large branches from it were seen
Figure 1. Occluded branches of the left middle cerebral artery (MCA, b) are shown in this figure. The lighter material seen within the vessels (arrow heads) is embolized atheroma. (ICA, internal carotid artery) × 15.

to be totally occluded. Grossly, the vessels were seen to be congested with blood and whitish material (fig. 1). In the group of animals given non-lethal doses there were no obstructed vessels observed.

Surface indications of encephalomalacia did not appear in any of the animals. However, in two of the surviving animals internal parenchymal lesions were observed in the coronal surfaces of sliced brains. These lesions were white in appearance and softer than surrounding parenchymal tissue. They were of variable size and appeared in the more posterior slices. A lesion in one of the animals showing motor deficit appeared to be in the region of formatio reticularis, according to the stereotaxic atlas of Monnier and Gangloff, and, therefore, would account for some of the gross motor disturbances observed in that animal. In the other animal, the left hemisphere and left cerebellum and medulla were soft upon removal of the brain. However, due to the extensive vascular congestion it was not possible to distinguish between non-perfusion of the fixative and true infarction.

Light microscopy of the brain slices demonstrated the general distribution and character of the vascular lesions. The major vessels at the base of the brain were clear after perfusion in animals given sublethal doses. In animals given lethal doses Circle vessels were congested as were one or more of its major tributaries on the ipsilateral side of injection.

The middle cerebral artery was most frequently occluded. Parenchymal vessels in both surviving and non-surviving animals showed similar regions of obstructed or congested vessels. These occluded vessels were found mostly, and randomly, throughout the cortical and subcortical parenchyma within the territory of the ipsilateral middle cerebral artery. Less frequently, occluded vessels were found in the ipsilateral anterior frontal lobe and, rarely in midbrain tissue. In two animals congested vessels were also found in contra-lateral cortical and subcortical parenchyma in the territory of the middle cerebral artery.

Cholesterol crystals from human atheroma having the typical rhomboid shape and angularity showed maximum dimensions of about 150 μ, (Unpublished data). Most observed, however, were much smaller, 20–50 μ, and irregular shape, due to fragmentation. This agrees with recent estimates by Steiner et al. Vessels found to be occluded by atheroembolic material (those with at least one visible cholesterol cleft in an occlusive mass) ranged in diameter from 10 μ in small parenchymal vessels to over 800 μ in some leptomeningeal vessels. These measurements excluded major vessels contributing to, of and branching from the Circle of Willis.

The character of the occluded vessels was usually that of congested vessels. However, many vessels, especially the leptomeningeal and penetrating vessels near the brain surface, contained atheroembolic occlusions typified by the presence of cholesterol clefs (fig. 2) as well as cellular and amorphous structures.

Generally, transmission electron microscopic examination of the lesions in both the larger vessels at the base of the brain and the smaller parenchymal lesions...
showed cholesterol-crystal spaces surrounded by varying amounts of fibrin, lipid and cellular debris, amorphous material and blood elements such as platelets, red cells and small numbers of various leukocytes (fig. 3). In some areas pericytes around occluded vessels were filled with inclusions of various morphology, perhaps either absorbed luminal contents or absorbed necrotic parenchymal material (fig. 4). Tissue samples taken from suspected areas of infarction at autopsy in the surviving animals also demonstrated vessels surrounded by phagocytic glial cells and pericytes.

Discussion

Ulcerated atheromatous material poses a serious threat to the patency of a target artery. Thus, ischemia or infarction could result in the parenchyma within its distribution. The most noted of the occlusive components are cholesterol crystals. These elements, if large enough, are unresolvable. Additionally, there is evidence that other materials within the atheroma are capable of provoking thrombotic and inflammatory reactions. These responses augment the likelihood of the development of larger and more unresolvable lesions. This is all the more threatening in cerebral atheroembolism, since the cerebral parenchyma can survive only minutes without the reestablishment of perfusion. Thus, in order to assess methods for reducing the threat from atheroembolic sequelae, a detailed understanding of the initial and early development process of atheroembolic lesions within a suitable animal model is highly desirable.

It is unlikely that immunological reactions contribute significantly to the development of occlusive lesions in this model. This belief is based on three considerations. Firstly, the morphology of lesions produced by this method have closely resembled experimentally induced lesions in other vascular beds and those described in the clinical literature. Secondly, each animal received only a single injection of the material. Finally, the results of Ochterlony plate tests have proven negative between samples of human atheromatous material and the serum of animals given sublethal doses of human atheromatous material. While it is possible for immunological reactions to influence thrombogenesis, it is not thought to be a major or even significant parameter in the development of these experimental lesions.

The quantitative results of this study would indicate that there is a specific load of whole atheromatous material which can be managed by the cerebral circulation of rabbits weighing between 2 and 3 kgs. Beyond this point sudden death usually occurs. The demonstration of a critical load could be interpreted as meaning that simple mass could be the deciding factor. However, having established a lethal dose of approximately 55 mg of whole human atheromatous material, the
injection of 80 mg of homogenized liver produced no observable symptoms or qualitative lesions. These results would suggest that the initial mass is not the only factor influencing the ultimate effect. Since the blood system can handle the liver material, presumably by an effective phagocytic response, then reaction(s) of the whole atheromatous material may augment the occlusive bolus or the effectiveness of the various patency maintaining mechanisms. The character of the lesions suggests that a potentially unmanageable thrombotic response occurs. Thus, a thrombogenic effect of the material could transform a mass which might have been pathogenically benign into one which becomes larger and more stable and possibly homeostatically unmanageable. A strong inflammatory response could further complicate an effective patency-maintaining response by both adding to the local congestion and possibly contributing to a thrombogenic response.

Oclusions of small vessels, for example at the arteriolar level, may have little effect, as compensatory mechanisms such as collateral circulation and patency maintaining systems such as phagocytic, fibrinolytic and prostaglandin (particularly PGL) responses may limit or inhibit any permanent loss of neuronal vitality. The occlusion of larger diameter vessels greatly increases the chance of more devastating and irreversible damage by reducing the availability of functional collateral vessels and limiting the accessibility of the patency maintaining mechanisms to sites of lesion development. It is frequently larger leptomeningeal vessels, and their cortical and subcortical tributaries, which are reported occluded in clinical descriptions of atheroembolic cerebral infarction; and in this model it was these vessels which became occluded in surviving animals. In non-surviving animals the Circle itself and frequently one or more of its major tributaries became congested. What we believe to occur is that as a vessel becomes occluded, there is a retrograde development of a thrombus. As in the case of Warren and Lytton, examining experimental limb artery lesions, an "ascending" (retrograde) thrombus can ultimately occlude a large vessel (the aorta in their case) and cause the death of the animal. In this model we suspect that the retrograde thrombus may fatally occlude large segments of the Circle.

Clinical descriptions of atheroembolic lesions are overwhelmingly of old lesions, usually of unspecified age. These are typified by the presence of one or more cholesterol clefts present in either an occluded vessel, with varying descriptions of cellular or amorphous elements associated with cleft; or recanalized vessels. Experiments in which the long term development of these lesions have been monitored have demonstrated these to be mature stages in the life of an atheroembolic occlusion. However, the few descriptions of early lesions have noted the presence of thrombotic material

**FIGURE 3.** This is an electron micrograph of a atheroembolically occluded and severely damaged cerebral parenchymal vessel. Aside from the presence of the acicular clefts and platelets, note the amorphous debris and the fibrin mass at the extreme left of the vessel lumen. × 6,000.
CEREBRAL ATHEROEMBOLISM \cite{Jeynes and Warren} and Warren

FIGURE 4. A pericyte with a number of inclusion vesicles is shown. Many of these were observed. The most frequent type of inclusion body was a membrane bound globule containing several small spheres of varying electron density. These may represent various kinds of absorbed lipids. Other inclusions were round homogeneous electron dense globules. \times 8,000.

Associated with the emboli, \cite{18, 19} Experimental studies in which human atheromatous material (HAM) was injected have also shown a thrombotic reaction in the resulting lesions, evidenced, as in this study, by the presence of fibrin and platelets within the lesions. \cite{18, 23, 35} It is interesting to note that some of the older literature has also reported the presence of inflammatory cells in acute early atheroembolic lesions. \cite{18, 27-30, 35}

Almost all of the work involving atheromatous material and the activation of the coagulation system has been done \textit{in vitro}. Although no specific provocative agents have yet been identified, many researchers have shown that atheromatous materials can provoke thrombogenic responses. \cite{23, 43-46} Inflammatory reactions have also been observed which demonstrate the presence of inflammatory lipids within atheroma. \cite{36} Two which are highly provocative are 26-hydroxycholesterol and cholestone 3β-5α-6β triol. Interestingly Greenberg et al. \cite{47} have linked atheroma cholesterol with the activation of complement and the aggregation of polymorphonuclear leukocytes (PMNs) in acute myocardial infarction. Even more interesting; elevated levels of cholesterol have been shown to enhance platelet production of thromboxane A2 (\textit{TxA2})\cite{48} PMNs can produce \textit{TxA2}\cite{49, 50} and PMNs have been shown to activate platelets. \cite{51} Further, the role of platelets in inflammation as a potential initiator\cite{52} and as an inhibitor\cite{53} of the reaction is currently under study. Thus, in the development of atheroembolic complications, several possible synergistic reaction scenarios are possible within the probable relationship between atheroma components, inflammation and thrombosis.

This animal model could be most useful in studying the reactions outlined above. Further, as a model for cerebral atheroembolic phenomena it could be useful for the study of cerebral infarction as well as one in which pharmacological agents could be tested for effective prophylactic treatment of the potentially more devastating sequelae of atheroembolism.

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