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MICROVASCULAR PERMEABILITY IN IRRADIATED RABBITS

by

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BELFRAGE & SCHILDT (1967) observed increased sensitivity to succinylcholine (SCh) in animals subjected to whole body irradiation while investigating the effect of SCh on the neuromuscular transmission in normal and irradiated rabbits. These experiments indicated that SCh had a cumulative effect in the latter. Among the possible mechanisms involved in this phenomenon, impaired peripheral circulation, secondary to microvascular irradiation injury was suggested as the cause. A subsequent investigation on the flow perfusion rate of muscle tissue in the hindlimbs of rabbits failed, however, to reveal any significant difference between irradiated and normal animals (BELFRAGE & SCHILDT 1968). The present investigation was therefore carried out to analyse further the microvascular response to irradiation, with special reference to the permeability of macromolecules.

Varying degrees of vascular damage have often been demonstrated in different tissues following irradiation (cf. p. 316). The permeability of the vessels of the peripheral nerves has been analysed following injuries other than those due to

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irradiation by, e.g. MELLICK & CAVANAGH (1967), OLSSON (1966), LUNDBORG (1970). The latter used fluorescence microscopy tracings of intravenously injected serum albumin, tagged with Evan's blue, to demonstrate changes of the permeability in microvessels of the rabbit's tibial nerve previously subjected to ischemia. This method has been used in the present investigation to evaluate the injury induced to endoneurial microvessels by irradiation. A slight, controlled ischemic injury was added to the limb to produce subliminal vessel injuries, not detectable by the tracer technique per se. This strategy was based on the findings of LUNDBORG (1970) that ischemia of 8 to 10 hours duration is necessary for causing damage to endoneurial vessels.

Material and Methods. The experiments were performed in 40 male rabbits (small chinchilla) 3 to 4 months of age and of about uniform weight (3.0 ± 0.4 kg). The experiments involved three procedures (cf. Table): (1) whole body irradiation of the rabbits, (2) induction of ischemia in one hind limb for 2 hours at intervals of 1 to 16 days, and (3) analysis of microvascular permeability in both hind legs subsequent to the ischemic period.

The animals were roentgen irradiated (Müller MG 300) two by two at 280 kV and 11 mA, the dose rate being 20 R/min with a total dose of 1 100 R, constituting approximately the LD_{50} for the strain (SCHILDT & SCHILDT 1963). The examinations were performed 1, 4, 8, 12 and 16 days after irradiation. The animals were anaesthetized intravenously with 30 mg/kg sodium pentobarbital (Nembutal) with additional small doses during the experiments. Before injection of the tracer substance, ischemia was induced in one hind limb for two hours by applying an infant's pneumatic cuff around the thigh of the rabbit and inflating it to about 700 mm Hg; this caused complete stagnation of flow in the intraneural microvessels as previously demonstrated by LUNDBORG (1970).

Changes in the vascular permeability of the tibial nerve were demonstrated by fluorescence microscopy tracings of intravenously injected serum albumin, tagged with Evan's blue. The tracer solution was prepared by mixing in vitro bovine serum albumin 5 % with Evan's blue by the method of STEINWALL & KLATZO (1965) and OLSSON (1966). The conjugate was carefully filtrated before use through a Sephadex column for removal of free tracer. The standard dose was 1 ml labelled albumin 5 % solution per 100 g body weight. This solution was slowly intravenously injected into an ear vein immediately after release of the cuff. The animals were killed 30 minutes after the injection by rapid infusion of an overdose of sodium pentobarbital (Nembutal) and specimens from the tibial nerve of both legs were taken for examination; the one from the leg subjected to both irradiation and ischemia will be called the experimental nerve and the other (irradiation only) the control nerve. The nerve specimens were

Table

Summary of investigation and findings

Group (n)	Treatment		Time after irradiation (days)	Petechial bleedings*	Increased permeability**
	Irradiation (R)	Ischemia (hours)			
A (7)	—	—	—	0	0
B (6)	—	2	—	0	0
C	1100	—	1	0	0
(4)	1100	2	1	+	+
D	1100	—	4	0	0
(6)	1100	2	4	++	+
E	1100	—	8	0	0
(7)	1100	2	8	+++	+++
F	1100	—	12	0	0
(5)	1100	2	12	+	++
G	1100	—	16	0	0
(5)	1100	2	16	0	0

* Grading of bleedings in skin and tibial nerve: 0 = not present, + = rare and spotted, ++ = = numerous but spotted, +++ = numerous and general.

** Grading of increased permeability as indicated by extravascular leakage of tracer: 0 = not present, + = moderate and spotted, ++ = moderate and general, +++ = massive and general.

fixed in formalin 5 % solution for 24 hours. Frozen longitudinal sections of 10 mμ thickness were mounted in aqueous glycerine 50 % and immediately examined in a Leitz fluorescence microscope equipped with a dark field condenser and an Osram HBO 200 W high pressure mercury lamp. The light was directed through a Schott BG 12/3 mm filter; the light emitted was passed in the tubes through a K 510 filter. Under these conditions, Evan's blue-albumin emits a bright red fluorescence (cf. the Figure), and intravascular or extravascular tracing of the conjugate may easily be performed.

Results

The results are summarized in the Table and Figure. The changes reported below were generally very much the same for each experimental group (A—G).

A. Unirradiated, non-ischemic control nerves (n = 7). No obvious blue staining was evident. Fluorescence microscopy: No extravascular red fluorescence was ever observed; accordingly no leakage of Evan's blue-albumin into the endoneurial space was evident (cf. Figure a).

B. Unirradiated, ischemic (2 hrs) control nerves (n = 6). No obvious blue staining of the nerves; no bleeding detected at any level. Fluorescence microscopy: No extravascular red fluorescence was apparent in the endoneurial space, the conjugate being strictly confined to the lumen of the endoneurial blood vessels (cf. Figure a).

C. One day after irradiation (n = 4). The nerve from the experimental limbs exhibited considerable blue staining and spotted bleeding of varying degree was occasionally apparent at varying levels along the nerve. The nerves from the control limb always presented normal appearances. Fluorescence microscopy: The experimental nerves displayed spotted extravascular leakage of tracer, the red fluorescence presenting an irregular diffuse distribution in the endoneurial space. Large areas of the nerve were, however, not subjected to the leakage; in these parts the red fluorescence was completely confined to the vessel lumen. The nerve from the control limbs never had any extravascular leakage of albumin.

D. Four days after irradiation (n = 6). The experimental nerves presented strong blue staining, and at all levels there were numerous areas of slight bleeding. The nerves from the control limbs always had normal appearances. Fluorescence microscopy: Like the nerves prepared 1 day after irradiation, diffuse red fluorescence was present at most levels in the endoneurium. Some areas did not, however, appreciably differ from the control nerves. The latter never presented any extravascular red fluorescence.

E. Eight days after irradiation (n = 7). These rabbits invariably had numerous spots of slight bleeding on both hind limbs in skin areas that had been subjected to hair-clipping prior to examination. The experimental nerve displayed numerous large areas of bleeding at all levels while no such phenomena were apparent in the control nerves. Fluorescence microscopy: Specimens from the experimental nerves had strong diffuse red fluorescence in all parts of the endoneurium, indicating a general leakage of albumin (cf. Figure c). The control nerves never had extravascular red fluorescence (cf. Figure b).

F. Twelve days after irradiation (n = 5). In two of the animals, scattered areas of bleeding occurred in the skin on limbs subjected to hair-clipping. Small areas of bleeding could occasionally be observed in the tibial nerve of the experimental side. The control nerve was normal in appearance. Fluorescence microscopy: Extravascular red fluorescence of varying intensity was apparent at all levels of the experimental nerves. The red fluorescence was confined to the lumen of the endoneurial vessels in the control nerves.

G. Sixteen days after irradiation (n = 5). No bleeding occurred in the skin on limbs subjected to hair-clipping, neither could any bleeding be observed in the tibial nerve of either limb. Fluorescence microscopy: No extravascular red fluorescence could be detected in the experimental or in the control nerves.

a) Control nerve from an untreated rabbit. The tracer (yellow-red) is strictly confined to the lumen of endoneurial blood vessels. Marked fibre structure of the fascicle (green).

b) Tibial nerve from left limb of a rabbit treated by total body irradiation 8 days previously. The limb was not subjected to ischemia before injection of the tracer. The Evan's blue-albumin is still strictly confined to the vessel lumen, no extravascular leakage being evident.

c) Tibial nerve from right limb of the same rabbit as in (b). The limb was further subjected to 2 hours ischemia before injection of the tracer. Marked leakage of tracer, the red fluorescence being diffusely distributed in the endoneurium.

Distribution of Evan's blue-albumin in the tibial nerve of rabbits under various experimental conditions as revealed by fluorescence microscopy.

Discussion

Interest has previously been focussed upon the behaviour of the endoneurial microvessels following various kinds of injuries (Olsson 1966, Mellick & Cavanagh 1967, Lundborg 1970). The effects of irradiation on the permeability of these vessels have, however, not been investigated. A new feature is the addition to the radiation injury of a second slight injury (in this case ischemia) which in itself is incapable of producing any detectable damage to the vessel walls. This method is thought to be a useful approach to problems concerning early microvascular changes. It also illustrates the important fact that two different types of injuries have a synergistic effect when combined (Schildt & Thorén 1968). The endoneurial vessels of the irradiated limbs, not being subjected to ischemia, failed to reveal any leakage of tracer albumin. In the legs kept ischemic for 2 hours, however, leakage was detectable as early as 24 hours following irradiation. The radiation damage could thus not be detected by the tracer technique per se but became apparent after the addition of a very slight endothelial injury corresponding to 2 hours ischemia. Since Lundborg (1970) has reported that as much as 8 to 10 hours ischemia is required to cause permeability disturbances, this short ischemia can in itself only have caused insignificant damage to the vessel walls.

Regarding the time interval between the exposure and the appearance of permeability disturbances, the present results indicate that an increased permeability is apparent on the 4th day, reaches a maximum around the 8th day and persists for about 2 weeks after irradiation. These findings are in line with several previous investigations that indicate the existence of a peak in microvascular permeability 1 to 2 weeks after exposure (Furth et coll. 1951, Wish et coll. 1952, Cronkite & Bond 1960, Várterész 1966, Arturson & Thorén 1968).

Considerable discrepancy exists regarding the exposure dose needed to inflict significant damage to the blood vessels. Generally speaking, the vascular tree is considered relatively radioresistant. According to Rhoades (1948), supralethal doses are required to produce recognizable structural disintegration of the endothelial cells. It has been stated that with doses around LD_{50}, the endothelium is rarely affected. Cronkite & Bond (1960) reported that irradiation doses of 1 500 R are necessary to cause detectable changes. From the present and from many previous investigations, however, it appears obvious that functional disturbances occur in the microvascular endothelium at considerably lower doses. Thus, doses corresponding to LD_{50} have been found to induce detectable general disturbances in microvascular permeability (Furth et coll. 1951, Wish et coll. 1952). Investigations involving histologic examination of the sciatic nerve in rabbits after local irradiation with 30 krad by Bergström (1962) revealed that the endothelium was still intact after 5 to 7 days.

The present investigations indicated that exposure to 1 100 R caused no obvious damage but the existence of a subliminal endothelial damage could be demonstrated by the addition of slight ischemia.

Several reports indicate a difference in radiosensitivity among arteries, veins and capillaries with regard to morphologic changes. LINSER (1905) could find no endothelial damage in the arteries or veins after irradiation, while the endothelium of the arterioles was either absent or swollen and projected into the lumen. On the other hand, LAZARUS-BARLOW (1922) reported that the arterioles were the only vessels escaping damage from gamma-irradiation. Another view was presented by EFSKIND (1940), who described radioresistance of endothelial cells in larger vessels but reported signs of cellular injury in the endothelium of smaller vessels. This view has been supported by others, e.g. RUBIN & CASARETT (1968).

The great variation in the results is apparently due to differences in the type, energy and dose of irradiation, the time lapse after exposure before examination, and species differences.

Most of the interest regarding radiation-induced damage to blood vessels has been focussed on the endothelium. Its swelling and proliferation, in certain conditions prominent enough to obliterate capillaries, have been reported (e.g. PORTER & WHITE 1907, WOLBACH 1925, ELLINGER 1935). CRONKITE & BOND (1960) also pointed to endothelial swelling as a characteristic feature together with degeneration of the smooth muscle and connective tissue coat of the blood vessels, particulary of the capillaries, such as increased permeability, have been demonstrated by several authors. PAINTER et coll. (1947), who injected Evan's blue into rabbits subjected to local irradiation, reported that the dye appeared more rapidly in irradiated than in non-irradiated areas. A rapid disappearance of labelled proteins, erythrocytes or Evan's blue from the circulation of irradiated (LD_{50}) rabbits has been reported by FURTH et coll. (1951) and WISH et coll. (1952). Increased vascular permeability was observed in dogs by CHERNOV et coll. (1965) as early as 24 hours after exposure to 600 R (LD_{100}) as indicated by an increased disappearance rate of intravenously injected fluorescein compared to controls. The permeability of the skin capillaries increased in dogs exposed to 500 R as reported by VÁRTERÉSZ (1966). A more sensitive method was used by ARTURSON & THORÉN (1968), who investigated the capillary permeability following local irradiation (200 to 4 000 R) of the paws of dogs by measuring the leakage of dextran molecules across the blood-lymph barrier. The draining lymphatics of the paws were cannulated and dextran of varying molecular size injected intravenously. The lymph to plasma concentration ratio (CL/CP) of the dextran was then determined. These authors found 'sieving characteristics' of the blood-lymph barrier in irradiated tissue to be correlated to both the ex-

posure dose and the time lapse after irradiation. The capillary leakage was greatest 2 weeks after irradiation. With 1 000 R, the CL/CP ratio was close to zero for dextran with a molecular weight of approximately 70 000, i.e. molecules of the same size as serum albumin failed to pass the capillary walls, which is in agreement with the present findings. The capillaries possessed, however, an increased permeability for smaller molecules as compared to non-irradiated controls. Larger and larger dextran molecules passed the blood-lymph barrier indicating definite damage in paws exposed to 200 to 4 000 R.

The non-specific effect of ionizing radiation should be emphasized. The microvascular reactions that characterize the early changes of inflammation are remarkably similar in different kinds of injuries and include vasodilatation, increased permeability and migration of leukocytes. Such changes may arise following for instance chemical injury, ionizing radiation, local anaphylaxis, mechanical trauma, thermal trauma, ultraviolet light injury (SCHILDT & ARTURSON 1970).

The considerable increase in vascular permeability to fluids, electrolytes and proteins may be due either to increased hydrostatic pressure in the microvessels or damage to the endothelial barrier itself. These changes may in turn be caused by physical effects or mediated by endogenous substances, released or activated by the injury.

Regarding the physical effects, three mechanisms are thought to govern the transfer of materials across the capillary membranes: (1) diffusion for small molecules, (2) ultrafiltration and more recently (3) pinocytosis for larger molecules. Larger molecules such as those of dextran or albumin are transported in bulk through a few capillary 'leaks' with a radius of about 250 Å (GROTTE 1965). The increased permeability after different injuries including irradiation is, according to ARTURSON (1970), due to an increase in the size of these leaks. This has been called the 'stretched pore phenomenon' and is localized anatomically to the venular side; it seems to act as a safety valve by letting out 'puffs' of the intravascular content into the extravascular compartment.

Damage to the intercellular substance is often given as an explanation of the increased capillary permeability present after irradiation as well as following other types of injuries. According to VÁRTERÉSZ (1966) and others, hyaluronic acid is depolymerized in vitro by irradiation already at low dose levels. It is split by hyaluronidase into glucuronic acid and acetyl-glucose amine. Irradiation activates hyaluronidase in vitro but final evidence that this occurs in vivo as well is at present lacking.

Most workers ascribe increased permeability chiefly to the existence of endogenous mediators. A great number have been suggested but only a few have stood critical examination. Among the latter are histamine, serotonin and various

proteases (WILHELM 1962, SCHACHTER 1969) and recently prostaglandin (ÄNGGÅRD et coll. 1970).

Conclusion

Whole body ionizing radiation around LD_{50} evidently may increase the permeability of microvessels to macromolecular substances. The nature of this is not clear, but resembles closely the increased permeability evident after other types of injuries. The reactions are nonspecific and apparently not governed by the type of injury in the first place, but rather by the ability of the exposed cells to react. The factors involved are (not in order of importance): leaks in the vessel wall, changes in the intercellular substance and the existence of endogenous mediators such as histamine, serotonin, proteases and prostaglandin.

SUMMARY

The microvascular permeability of the endoneurial vessels of the tibial nerve was investigated in rabbits exposed to 1 100 R roentgen irradiation, corresponding to LD_{50}. Radiation damage was not detected following the tracer technique per se. The addition of slight ischemic injury, not capable in itself of causing permeability disturbances, revealed however vascular damage during the first two weeks following irradiation.

ZUSAMMENFASSUNG

Die mikrovaskuläre Permeabilität der endoneuralen Gefässe des Nervus tibialis des Kaninchens wurde nach Bestrahlung mit 1 100 R Röntgenstrahlung entsprechend der LD_{50} untersucht. Der Strahlenschaden war per se mit Hilfe der Spurentechnik nicht nachweisbar. Wurde zusätzlich ein leichter ischämischer Schaden hinzugefügt, der selbst nicht zu Permeabilitätsänderungen führte, wurde jedoch ein klarer vaskulärer Schaden während der zwei ersten Wochen nach der Bestrahlung deutlich.

RÉSUMÉ

Les auteurs ont étudié la perméabilité microvasculaire des vaisseaux endoneuraux du nerf tibial sur des lapins après une irradiation par 1 100 R de rayons de Roentgen, correspondant à une LD_{50}. Cette technique de traçage n'a pas permis à elle seule de détecter les lésions dues à l'irradiation. L'addition d'un léger dommage ischémique, incapable par lui même de causer des troubles de la perméabilité, a cependant révélé une nette lésion vasculaire au cours des deux premières semaines après l'irradiation.

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