PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Vascular Injury: Platelets and Smooth Muscle Cell Response [and Discussion]

M. B. Stemerman, I. F. Skidmore, J. R. O'Brien and Elspeth B. Smith

Phil. Trans. R. Soc. Lond. B 1981 **294**, 217-224
doi: 10.1098/rstb.1981.0100

Phil. Trans. R. Soc. Lond. B **294**, 217–224 (1981)
Printed in Great Britain

Vascular injury: platelets and smooth muscle cell response

By M. B. Stemerman
Department of Medicine, The Charles A. Dana Research Institute and The Thorndike Laboratory of the Harvard Medicical School, Beth Israel Hospital, 330 Brookline Avenue, Boston, Massachusetts 02215, *U.S.A.*

The blood platelet appears to play an important role in the pathogenesis of the atherosclerotic plaque. Using a platelet specific antigen, platelet factor 4 (PF4), we have demonstrated that PF4 released from platelets enters the vessel wall. Smooth muscle cell (s.m.c.) proliferation *in vivo* was examined by using a new technique for measuring [^{3}H]thymidine incorporation. With this technique, we have shown that a remote vascular injury can cause s.m.c. proliferation, presumably mediated by a humoral agent. Endothelial dysfunction, in turn, may be caused by a sustained, mild hypercholesterolaemia. This permeability dysfunction may provide circulating s.m.c. mitogens with access to the vessel, wall, thus allowing s.m.c. proliferation in areas of non-desquamated endothelium. These experiments form the basis for a modification of the hypothesis implicating platelets in atherogenesis.

The vascular smooth muscle cell (s.m.c.) and the blood platelet have been closely linked with atherosclerotic plaque formation. Although the contribution of platelets to atherosclerotic plaque build-up has been discussed for many years, it has only recently been suggested that platelets may play a causative role in vascular s.m.c. proliferation (Ross & Glomset 1976). This platelet effect is of particular importance, since the s.m.c. is the principal cell of the atherosclerotic plaque (Wissler 1968). The recent interest in this platelet–s.m.c. interrelation has been stimulated by the finding obtained from the propagation of vascular s.m.cs in cell culture. Cell culture experiments with the use of s.m.cs have demonstrated the s.m.cs ability to produce extracellular connective tissue including collagen and elastin, to metabolize lipoproteins and to be stimulated to grow by a mitogen carried by platelets.

The study of s.m.c. biology in culture is limited by the rapid metabolic changes that the cells undergo once placed in culture (Fowler 1977) and hence the potential change in their nature. On the other hand, characterization of s.m.c. growth *in vivo* has been hampered by difficult methodological problems. The use of autoradiography, for instance, is tedious and time-consuming. To overcome this problem, we have established an *in vivo* technique for determining s.m.c. proliferation based upon the incorporation of [^{3}H]thymidine into s.m.c. DNA after de-endothelialization of the aorta by a balloon catheter (Stemerman 1973). The results of these experiments demonstrate that the method is rapid, quantitative and can be used to determine vascular s.m.c. growth.

The link between platelets and s.m.cs rests on the hypothesis that platelets carry an s.m.c. mitogen that can be released from the platelet α-granule and can enter the vessel wall at sites of injury (Stemerman 1979). Platelets do indeed attach to subendothelial connective tissue after endothelial desquamation, but little is known of the release of their intraplatelet contents

and whether these materials can penetrate into the vessel wall. To trace the products of platelet release, we have purified a platelet-specific protein, platelet factor 4 (PF4) in rabbits and have raised an antibody against this antigen (Goldberg *et al.* 1980*b*). Using immunofluorescent microscopy, we have been able to detail the course of PF4 when released from platelets and have investigated potential inhibitors of the platelet release reaction *in vivo*.

Smooth muscle cell proliferation *in vivo*

For an examination of s.m.c. proliferation *in vivo*, rabbits were de-endothelialized by balloon catheter (Stemerman 1973) and injected with [^{3}H]thymidine, (0.5 μCi/g body mass) (20 mCi/mmol; New England Nuclear) intravenously (i.v.), followed 30 min later by an i.v. injection of 5 ml of Evans blue dye (Harvey Labs. Inc.). Evans blue was given to assure endothelial removal: the dye penetrates areas of intima not covered by endothelium. Rabbits were killed by cardiac exsanguination at 1, 8, 16, 24, 36 and 48 h and at 4, 5, 6, 7, 14, 28 and 48 days after initial injury; 5–12 rabbits were used at each interval sampled. Within 1 min after exsanguination, a thoracic segment (third to sixth intercostal arteries) and an abdominal segment (left renal to the aortic bifurcation) were removed, immediately frozen and stored at −70 °C. These segments were used to determine DNA specific activity as outlined below. Adjacent thoracic and abdominal segments were not frozen but were immediately immersed in 2.5% glutaraldehyde (0.1 M cacodylate, pH 7.4, 20 °C) and processed for morphological and morphometric evaluation. The frozen segments from each rabbit were processed separately. The vessels were thawed and the adventitia was stripped away from each segment and discarded. The remaining intima–media tissues were homogenized and the Evans blue extracted by using heparin–Sepharose beads. DNA content was determined on the solubilized segments by the method of Burton. Samples of solubilized tissues were precipitated in parallel at 4 °C for 30 min in 10% trichloroacetic acid (TCA) and bovine serum albumin. Precipitates were trapped on a nitrocellulose filter, solubilized with methoxyethanol and their radioactivity determined in aquasol scintillation fluid (New England Nuclear). DNA specific activity was calculated for each sample, and the means ± standard errors were plotted against time (Goldberg *et al.* 1980*a*, 1979).

The fixed thoracic and abdominal segments were each cut into 1 mm rings, post-fixed in osmium tetroxide and stained with uranyl acetate for 30 min. The rings were dehydrated and embedded in Epon. Epon sections (1 μm) were cut from each of the thoracic and abdominal blocks of each animal and stained with methylene blue. These sections were evaluated for intimal cell nuclei and by radioautography for grain counts.

Results of the experiment indicate that the thoracic aorta, in comparison with the abdominal aorta, shows much less increase in DNA specific activity (figure 1) and likewise there is much less intimal s.m.c. accumulation (table 1). Thymidine incorporation into s.m.c. DNA does not begin until 24 h after de-endothelialization. At 24 h, a steady rise in DNA specific activity begins in both the abdominal and thoracic segments, peaking at days 2–4 and then gradually returning to the baseline by approximately 28 days. Radioautographic studies carried out in parallel with measurements of DNA specific activity show a correlation of $r = 0.77$.

These data indicate that, in response to a similar injury, s.m.cs from the abdominal aortic segment proliferate at a greater rate than those of the thoracic region. Segmental variation may therefore be a common feature of s.m.c. proliferation *in vivo* and may account, in part, for

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

differences in growth of s.m.cs and hence the different sizes of plaques in different areas. In subsequent studies, de-endothelialization by balloon was carried out again at day 4 after the initial de-endothelialization. Injury was inflicted only on the abdominal aorta. Thoracic aortic segments (not subjected to the second injury) were analysed for [^{3}H]thymidine incorporation into DNA after this injury at days 4.5, 5, 6 and 7. There is a marked increase in [^{3}H]thymidine

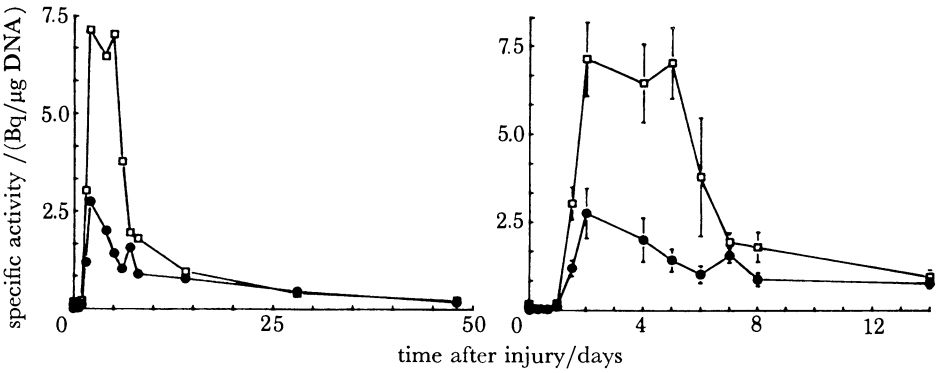

FIGURE 1. DNA specific activity curves for incorporation of [^{3}H]thymidine into aortic smooth muscle cells. There is a latent period of 24 h followed by uptake of the label. Peak activity occurs 2–4 days after injury with a gradual decline in uptake. There is a marked difference in the DNA activity between thoracic (●) and abdominal (□) segments. This is seen most dramatically at days 2 and 4. Student's *t*-test comparing thoracic and abdominal segments showed a value of $p < 0.01$. (1 Bq = 60 disintegrations/min)

TABLE 1. INTIMAL CELL NUCLEI: ABDOMINAL AND THORACIC SEGMENTS

(Intimal cell numbers ± standard error; number of samples shown in parentheses.)

day	abdominal	thoracic
1	1.2 ± 0.20 (12)	0.81 ± 0.028 (9)
2	1.1 ± 0.17 (12)	0.21 ± 0.027 (12)
4	2.3 ± 0.39 (12)	0.66 ± 0.12 (12)
7	11.6 ± 0.74 (9)	7.38 ± 0.49 (17)
14	20.5 ± 0.75 (12)	11.0 ± 1.06 (11)
28	21.8 ± 1.71 (6)	11.3 ± 1.02 (6)
48	30.8 ± 0.46 (9)	13.9 ± 0.85 (9)

incorporation in the non-reinjured thoracic segment compared with the control animals. There is a peak specific activity at day 5 of approximately 30 Bq/mg DNA. These latter experiments indicate that a distal vascular injury, potentially freeing platelet materials into the circulation, can cause s.m.c. proliferation at a different vascular segment not experiencing the injury. This observation implicates a humoral material involved in smooth muscle cell proliferation that is effective *in vivo*. Whether this material is indeed platelet-derived is as yet unknown.

PLATELETS AND THE VESSEL WALL

To allow the course of materials released from platelets as they interact with the vessel wall to be followed, PF4 was purified from rabbit platelets. A monospecific antibody was raised in a goat against this antigen and used to detect the presence of rabbit PF4 antigen. Rabbit iliac arteries were denuded of endothelium with a low-pressure balloon catheter (Stemerman 1973). At 10 min, 30 min and 4 h after de-endothelialization, the animals were killed by exsanguina-

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

tion and portions of the injured artery were immediately frozen. These arteries were sectioned after freezing and were reacted with the anti-PF4 antibody by indirect immunofluorescent techniques adapted for the PF4 antigen (Goldberg *et al.* 1980*b*).

The PF4 antigen was identified at the luminal surface and in the tunica media of the artery 10 and 30 min after removal of the endothelium. Immunofluorescent platelets were seen on the vessel surface at 10 min. At this time, penetration of the antigen into the vessel wall was noted in the inner luminal one-third of the vessel. Platelets remained at the surface 4 h after injury and showed fluorescence, but there was virtually no immunofluorescent staining of the vessel wall by this time. Control arteries not injured showed no fluorescence in the vessel wall. The anti-PF4 serum was absorbed by incubation with purified PF4 protein. Application of the absorbed serum to vessel frozen sections produced no immunofluorescence. These results are consistent with those of morphological studies of platelet attachment to the vessel wall, and provide additional information about the reaction between platelets and the vessel wall several hours after injury. They also agree with the results of recent studies of ^{51}Cr label platelet turnover (Groves *et al.* 1979). In those studies, platelet interaction with the vessel wall was evaluated in rabbits whose endothelium was removed in the balloon catheter. It was shown that only 0.2 % of the circulating platelets attached to the damaged wall and that turnover was almost undetectable. Similarly, we have shown that secretion of antigen by platelets is a short-lived, self-limited phenomenon, with apparently little secretion occurring 4 h after injury.

Additional studies have been performed by using PF4 antigen as a probe for platelet release during a constant intravenous infusion of prostacyclin (PGI_2) (Adelman *et al.* 1980). Rabbits were given large doses (800 ng kg^{-1} min^{-1}) of PGI_2. In these rabbits, their platelets, tested by aggregometry, were non-reactive to collagen and adrenalin. Animals were killed 30 min after de-endothelialization by ballooning and the vessels removed and studied for the presence of PF4 antigen. A second group of rabbits was given a constant infusion of saline as control. The platelets in this second group adhered normally to the denuded subendothelium by light and electron microscopy and PF4 was identified by immunofluorescence within the vessel wall. In contrast, under a constant infusion of a large dose of prostacyclin, platelet adhesion to the vessel wall was greatly reduced and PF4 antigen was identified at occasional sites of attachment of rare, randomly distributed platelets on the subendothelium. Of importance is the observation that little if any PF4 antigen traversed the vessel wall. Large doses of prostacyclin inhibit release of PF4 antigen into the vessel wall.

Endothelial injury

A basic tenet of atherogenesis states that endothelial cells (e.cs) protect against intimal proliferation by providing the vessel wall with a non-thrombogenic surface and a permeability barrier. The mechanism for the non-thrombogenic quality of the endothelium remains unclear but has been considered to be associated with the endothelial cells' ability to produce prostacyclin (PGI_2). The e.c. forms an effective barrier to penetration of materials into the vessel wall, limiting permeation into the underlying tissue to concentrations approximately one-hundredth that of materials in the plasma (Stemerman 1979). With removal of the endothelium, there is loss of this non-thrombogenic permeability shield, which (*a*) produces a haemostatic response to the now-exposed thrombogenic subendothelium, and (*b*) allows the unrestrained entrance of plasma constituents into the vessel wall. It has been considered that endothelial

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

cells, when damaged, would be shed from their vascular surface. To test this hypothesis under a mild hypercholesterolaemic stress, we fed a cohort of rabbits a diet of their normal rabbit feed supplemented with whole eggs. The animals were fed this diet for 7 weeks, reaching a mean serum cholesterol level of 451 mg/dl. The animals were then killed by perfusion fixation (Stemerman 1973), but 1 min before perfusion they were given an intravenous infusion of horseradish peroxidase (HRP) (Sigma, type II), 80 mg/kg, over a 10 s period. The perfused

TABLE 2. INCREASE IN AREA OF INTIMA STAINED BY HORSERADISH PEROXIDASE AFTER 7 WEEKS OF HIGH-CHOLESTEROL DIET

(Mean serum cholesterol increased from 59 ± 3.2 to 451 ± 29.8 mg/dl.)

diet	percentage area stained ± s.e.m.	number
normal	12.1 ± 5.0	6
egg-supplemented	45.6 ± 6.2	7

Comparison of group by Student's t-test: $p < 0.01$.

arteries were pinned out and reacted with diaminobenzidine–H_2O_2 in the dark. The surface was examined *en face* and showed 1–2 mm spots of brown at the endothelial surface indicating heightened permeability of the HRP permeability marker in these areas. The area of heightened permeability was calculated by morphometrics (Weibel 1973) and compared with a control group of rabbits not supplemented with eggs. As seen from table 2, there was a marked increase in the area of heightened permeability in the hypercholesterolaemic animals. These heightened permeability areas were examined by scanning electron microscopy (s.e.m.) and transmission electron microscopy (t.e.m.). S.e.m. showed some distortion of the linear arrangement of the endothelium; T.e.m. showed only increased penetration of the reaction product of the HRP but no qualitative changes in the e.c. ultrastructure. Neither t.e.m. nor s.e.m. showed loss of endothelium or platelet accumulation at the site of these permeability defects. The cause of the heightened permeability is not clear but it is apparent that loss of endothelium is not necessary for heightened permeability to occur. In the case explored, a mild elevation of serum cholesterol concentration caused a change in permeability of the vessel wall due to an 'endothelial dysfunction' without endothelial desquamation (Stemerman 1981).

A MODIFIED HYPOTHESIS

The studies provide a basis for modifying the current hypothesis that links platelets to s.m.c. proliferation. Basic to that hypothesis is the tenet that endothelial injury will lead to endothelial desquamation. After endothelial detachment, the desquamated surface is available to the blood as a thrombogenic surface. Platelets can adhere to this surface and can release intracytoplasmic constituents into the vessel wall. As seen by the above experiments, e.cs may become dysfunctional in the face of a sustained modest hypercholesterolaemia but do not desquamate. A dysfunctioning cell would allow the access of greater concentrations of circulating materials into the underlying vessel wall. Thus, mitogenic materials that might be constituents of plasma or might be released by platelets intravascularly, for whatever reason, could accumulate sufficiently in the vessel wall to initiate s.m.c. proliferation under an intact endothelium. It

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

appears, from our studies on segmental s.m.c. responses to injury, that certain s.m.c. beds are much more responsive than others Combined with an area of endothelial dysfunction, such areas may be more disposed than others to proliferate. Since our studies have demonstrated that a vascular injury can cause an s.m.c. proliferative response in a non-injured segment, this pathway may give rise to atherosclerotic lesions in susceptible areas over extended periods. A course of intervention in this sequence of events would be directed toward reduction of risk factors such as elevated serum levels of cholesterol, which cause endothelial dysfunction along with suppression of platelet function.

This work was supported in part by grants no. HL 25066 and HL 22602 from the National Institutes of Health, Bethesda, Maryland.

References (Stemerman)

Adelman, B., Mannarino, A., Goldberg, I. D., Stemerman, M. B. & Handin, R. 1980 Prostaglandin I_2 prevents platelet interaction with injured vessels. *Clinical Res.* **28**, 489A.

Fowler, S., Shio, H. & Wolinsky, H. 1977 Subcellular fractionation and morphology of calf aortic smooth muscle cells. Studies on whole aorta, aortic explants and subculture grown under different conditions. *J. Cell Biol.* **75**, 166–184.

Goldberg, I. D., Stemerman, M. B. & Handin, R. I. 1980*a* Vascular permeation of platelet factor 4 after endothelial injury. *Science, N.Y.* **209**, 611–612.

Goldberg, I. D., Stemerman, M. B., Ransil, B. J. & Fuhro, R. I. 1980*b* *In vivo* aortic muscle cell growth kinetics: differences between thoracic and abdominal segments after intimal injury in the rabbit. *Circuln Res.* **47**, 182–189.

Goldberg, I. D., Stemerman, M. B., Schnipper, L. E., Ransil, B. J., Crooks, G. W. & Fuhro, R. L. 1979 Vascular smooth muscle cell kinetics: a new assay for studying patterns of cellular proliferation *in vivo*. *Science, N.Y.* **205**, 920–922.

Groves, H. M., Kinlough-Rathbone, R. L., Richardson, M., Moore, S. & Mustard, J. F. 1979 Platelet interaction with damaged rabbit aorta. *Lab. Invest.* **40**, 194–200.

Ross, R. & Glomset, J. 1976 The pathogenesis of atherosclerosis. *New Engl. J. Med.* **295**, 369–375.

Stemerman, M. B. 1979 Hemostasis, thrombosis and atherogenesis. In *Atherosclerosis reviews* (ed. A. M. Gotto & R. Paoletti), vol. 6, pp. 105–146. New York: Raven Press.

Stemerman, M. B. 1973 Thrombogenesis of the rabbit arterial plaque: an electron microscopic study. *Am. J. Path.* **73**, 7–26.

Stemerman, M. B. 1981 Effects of moderate hypercholesterolemia on rabbit endothelium. *Arteriosclerosis* **1**, 25–32.

Weibel, E. R. 1973 Stereologic techniques for electron microscopic morphometry. In *Principles and techniques for electron microscopy*, (ed. M. A. Hayat), vol. 3, p. 237. Rheinhold, N.Y.: Van Nostrand Press.

Wissler, R. W. 1968 The arterial medial cell, smooth muscle or multifunctional mesenchyme? *J. Atheroscler. Res.* **8**, 201–213.

Discussion

I. F. Skidmore (*Glaxo Group Research, Ware, Herts., U.K.*). Having mentioned the platelet-derived growth factor of Ross, would Professor Stemerman comment on the experiments of Gospodarowicz & Tauber (1980), which suggest that mitogenic factors present in serum but not in plasma, and thus presumably of platelet origin, are only required when smooth muscle cells are grown on synthetic surface, and that when smooth muscle cells are grown on extracellular matrix the mitogens contained in plasma are adequate. Secondly, the same workers have shown that HDL is mitogenic for vascular endothelial cells while LDL is toxic at physiological concentrations. Does Professor Stemerman think that this is significant in the response to vascular injury?

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Reference

Gospodarowicz, D. S. & Tauber, J.-P. 1980 The control of vascular endothelial and smooth muscle cells by lipoproteins, FGF and the extracellular matrix. Presented at Symposium on Cellular Interactions, Cambridge, 23–26 September.

M. B. Stemerman. The experiments by Gospodarowicz & Tauber suggest that the cellular matrix provides the necessary information to eleviate the serum requirement for s.m.c. growth *in vitro*. Considerable s.m.c. growth was observed in the presence of plasma if an endothelial cell extracellular matrix was employed in the culture system. These experiments can be interpreted to suggest that the matrix is trapping serum-derived or platelet-derived growth factors. The ^{125}I-labelled FGF derivative used as a control in these studies is primarily basic myelin protein (BMP) as recently described, and if FGF can be iodinated among the BMP, the specific radioactivity of the ^{125}I-labelled FGF is probably very low. In addition, although ^{125}I-labelled FGF may be available, no evidence was presented to suggest that the ^{125}I-labelled derivative is biologically active while bovine brain FGF possesses an acidic p*I*, which eliminates it as a molecule for control. It is unfortunate that neither ^{125}I-labelled platelet-derived growth factor (PDGF) nor ^{125}I-labelled insulin-like growth factor-I (IGF-I) were used since both cationic hormones have been purified and the biological activity of the ^{125}I-labelled derivative assessed. The same logic applies to the question concerning HDL compared with LDL.

J. R. O'Brien (*Central Laboratory, St Mary's Hospital, Portsmouth, U.K.*). I was fascinated to see the amount of immunofluorescence in the sub-endothelium of the ballooned aortae indicating extensive PF4 deposition. Pepper and coworkers (Pumphrey *et al.* 1979) have shown that after minute doses of heparin IV, e.g. 500 U, there is a massive release of PF4 from some depot, but presumably not from platelets since there is no parallel release of βTB. An obvious depot was the endothelium or subendothelium. Accordingly I ask Professor Stemerman if animals were exposed to any heparin, and if not, what he thinks would happen to heparinized animals that were ballooned, or ballooned animals that were subsequently heparinized.

Reference

Pumphrey, C. W., Pepper, D. S. & Dawes, J. 1979 Heparin-PF4 pulsing as a measure of platelet endothelial cell reaction *in vivo*. *Thromb. Haemostas.* **42**, 43.

M. B. Stemerman. The animals described in our experiments were not given heparin. It is indeed possible that PF4 would bind to heparin and not enter the vessel wall; however, since no experimental data are yet available, we can only speculate on this.

Elspeth B. Smith (*Department of Chemical Pathology, University Medical Buildings, Foresterhill, Aberdeen, U.K.*). Caution is needed in interpreting endothelial injury in terms of allowing LDL to flood into the subendothelial space. In normal human aortic intima, the concentration of LDL, on a crude volumetric basis, is actually twice the concentration in the patient's plasma. Thus, removing the endothelium should in theory reduce the concentration of intimal LDL by allowing it to re-equilibrate with plasma.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

May I ask Professor Stemerman a question about the [^{3}H]thymidine labelling. There was a sharp bump in the specific activity curve just before the number of s.m.cs increased steeply and then settled at a constant high level. What happened to the actual rate of cell division? Did it remain high?

M. B. Stemerman. Recent studies by Dr Minick show that apolipoprotein B appears to be selectively trapped in areas that correspond to glycosaminoglycan deposition. The role of LDL in the pathobiology of smooth muscle cell proliferation *in vivo* remains unclear since its availability to the vascular s.m.c. is unknown. Therefore, not concentration of LDL but rather availability to the s.m.c. is the critical factor.

The growth rate of smooth muscle cells peaks at approximately day 7 (Goldberg *et al.* 1980*b*).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES