

THE EFFECT OF A LABORATORY ENVIRONMENT ON GRAFT REJECTION
IN LACERTA VIRIDIS, THE EUROPEAN GREEN LIZARD

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ABSTRACT Lacerta viridis received skin auto- and allografts. In laboratory conditions the allograft rejection response was very slow (220 - 320 days) or absent. In a more natural outdoor environment allograft rejection occurred in all lizards, grafts being rejected much faster (32 - 64 days).

INTRODUCTION

In 1971, Cohen reviewed "our rather limited knowledge of reptilian immunity" (1). At that time most immunological work concerning reptiles dealt with the Chelonia, while the Squamata, Crocodylia and Rhynchocephalia had been the subject of relatively few investigations.

The lizards and snakes are the most abundant modern reptiles, both in numbers of species and in numbers of living individuals (2), but with the notable exception of the comprehensive programme of research into the immune system of the agamid lizard Calotes versicolor (3,4,5,6,7), only a limited number of observations has, even now, been made on the immunology of this group. As a result, even the "base line data on the reptilian immune response" (1) are available only for C. versicolor, in the Squamata. We decided, therefore, to investigate aspects of the immune response in another species of lizard to try to complement the Calotes studies.

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Reptiles reject allografts in a chronic manner, but the details and timing of loss of graft colour, signs of necrosis in the graft tissue, inflammation, lymphocyte accumulation and sloughing of the graft vary from species to species (3,8,9,10,11,12,13,14,15). Since the review by Borysenko (15) of transplantation immunity in reptiles, further reports on skin allograft rejection have appeared, including work on parthenogenetic species and on snakes (9,11,14,16, Table 1). The use of allograft rejection as an indicator of genetic identity in suspected parthenogenetic lizard populations has provided some new data (9,11,14), but these were primarily studies of parthenogenesis, not of immunological responses to allografts.

This paper describes skin transplantation experiments in Lacerta viridis, the European green lizard, and the effect of different environments on graft rejection.

MATERIALS AND METHODS

Animals

L. viridis is one of about 180 species of the family Lacertidae, the "typical lizards" of Europe, Africa and Asia. Of the 51 species of lizard in Europe, 38 are lacertids (17).

L. viridis were purchased from commercial suppliers in England. They had been collected from a number of localities in Rumania and shipped to England via Italy.

Environment

One group of lizards (these will be called 'indoor') was housed indoors in ventilated plastic tanks (220 x 420 x 260mm) at a density of 1 or 2 per tank. The temperature was maintained between 22 and 30°C. They were fed on house crickets and mealworms, with supplementary vitamins (Vionate, E.R. Squibb & Sons, Ltd., Twickenham, England). Water was freely available. The lizards weighed 17 - 24 g and measured 85 - 120mm from snout to vent. All were mature, adult lizards. Males were slightly larger than females. Light was provided by 'Trulite' fluorescent tubes, which have enhanced emission at the blue end of the visible spectrum.

A second group of lizards (called 'outdoor') was housed in a large outdoor enclosure situated in an area of parkland at Essex University providing cover, sunlight and freedom from disturbance or predation.

A reservoir was included in the enclosure and this allowed natural precipitation to maintain a supply of water. The lizards fed from the natural population of insects and crustaceans in the enclosure and this supply was supplemented with crickets. The enclosure provided no barrier to animals smaller than the largest insects.

Skin transplantation

Anaesthesia was induced in L. viridis by intraperitoneal injection of sodium pentobarbitone (2.5 mg per 100 g body weight); induction required 3 - 30 minutes and recovery 3 - 12 hours.

Pieces of skin measuring about 5 x 5 mm were cut from the animals' dorso-lateral surfaces. Grafts were cut through the entire thickness of the skin. Sterile scissors, scalpels and forceps were used, but otherwise no attempt was made to disinfect the wound area. Care was taken to disturb the skin and wound as little as possible. All grafts to be transferred at one time were cut and detached from the graft beds and then left in position on the animals until all grafts had been cut, thus the actual transfer of the grafts could then be effected quickly. Grafts adhered to the graft beds without suturing, dressings or adhesives. Elasticity of the skin sometimes expanded the graft beds, so grafts were always arranged with at least two edges in contact with those of the host's skin. The lizards were kept isolated after grafting to avoid damage to the newly adhering tissue.

The method used for determining the sequence of allograft donors and recipients followed that of Maslin (8), but the maximum number of grafts per lizard was 6, comprising one autograft and up to 5 allografts.

Melanophore examination

Melanophores were observed in scales prepared by a method based on that of Manickavel & Muthukkaruppan (3). The tips of scales were removed from graft and control (undisturbed) areas at intervals, fixed in Heidenhain's Susa fixative, cleared in methyl salicylate - xylene and examined as whole mounts without staining.

Skin sections

Pieces of skin larger than, and containing, the grafts were removed from the animals and fixed as above. Paraffin sections 5 - 7 μ m were stained with Wälgert's iron haematoxylin and eosin.

Environmental records

Temperature in each environment was measured continuously with a recording thermometer. Records of rainfall, sun, humidity and temperature

were obtained from a weather station in the area.

RESULTS

All lizards

Grafts successfully adhered to the graft beds and then healed well. Sections cut at this stage (around 10 days) showed allografts indistinguishable from autograft controls. Autografts remained healthy and in the same condition as the surrounding skin for periods beyond 360 days. Melanophores in scale-tip whole mounts from autografts had a similar stellate structure to those in scales from untouched skin. Such normal melanophores showed variable morphology, so that the appearance of stellate structure, or its disappearance, was not considered reliable as evidence for acceptance or rejection of a graft, since a wide range of morphology (from large stellate to small and granular) could be observed in normal scales and often in different parts of a lizard at one time.

The criteria used to assess whether a graft was undergoing rejection were: raising of graft surface; large aggregations of pigment in scale tip whole mounts; centripetal loss of the normal green colour of the scales of the graft. The end-point for rejection was taken as sloughing of the graft.

In grafts undergoing rejection, loss of colour occurred centripetally, bright green giving way to dull black, and the surfaces of the scales became flaky. We observed a change in the organisation of the melanophores: scales from grafts undergoing rejection contained large aggregations of pigment, unevenly distributed through the scale. The surface of the graft was raised above that of the surrounding skin, but, as in the autografts and non-rejected allografts, histological examination failed to show accumulation of mononuclear cells.

Grafting on 'indoor' lizards

In lizards kept indoors responses to allografts were variable; the behaviour of 128 allografts on 52 lizards is summarised in Table 2. Most allografts were accepted as effectively as autografts. In all these cases skin colour remained normal over the surface of the grafts, there was no inflammation and the graft tissue effectively took the place of the original skin. No cellular infiltration of the graft tissue was observed. Only two allografts were rejected in this group, and those after a very

prolonged period. In none of these cases was a graft actually sloughed before the end of the observation period. However, in the lizards showing the most active allograft rejection, the graft was eventually loose enough to be easily removed with forceps, uncovering an area of new skin which was already well developed. Sections through the graft bed suggested that rejection had been effectively completed before this stage. The important feature of these results is that less than two per cent of allografts were rejected in 360 days of observation.

Grafting on 'outdoor' lizards

In lizards kept in the outdoor enclosure the number of grafts rejected and the rejection times were markedly different from those observed with the 'indoor' lizards. 13 lizards each had 2 allografts and 1 autograft. All the allografts were rejected by 64 days after grafting. The same signs of rejection were observed, but with correspondingly shorter time intervals between the start of rejection and sloughing. The results of all the allografting studies are summarised in Table 3.

DISCUSSION

Environmental conditions profoundly influenced the response of L. viridis to skin allografts.

In lizards kept indoors allograft rejection, when it occurred, was very slow and was similar to that observed by Cuellar & Smart in Cnemidophorus tigris where the "gradual" type of allograft rejection took up to 350 days (9). Their study is the only one to use a laboratory environment in which both light and temperature were controlled to provide some simulation of natural conditions. In part of our study (the 'indoor' group) lizards were kept at a relatively constant temperature: high temperatures (in this area this means up to 30°C) were recorded only rarely, on summer days when the outside temperatures were also high (Fig. 1a). This temperature control does not simulate the natural conditions to which this species is exposed, even though the normal temperature range has a similar mean to that maintained in the laboratory.

The lack of active allograft rejection reactions could have been due to genetic similarity between hosts and donors, but this question cannot be answered for a wild-caught group of unknown origin. There can be no likelihood, however, of genetic identity as in the parthenogenetic

Cnemidophorus tessellatus or Lacerta saxicola (8, 10). However, if our L. viridis had been collected in large numbers from restricted areas by the removal of inactive animals from their places of refuge at the end of hibernation - this is one method of collection used - then they could belong to inbred subpopulations. Individuals could in that case show the range of allograft rejection activities which we observed.

We therefore inferred from our results that there were three possible reasons for the poor allograft rejection response in the 'indoor' lizards:

a) The cell-mediated allograft response in L. viridis is poor, or, b) the animals had similar genotypes, or, c) the conditions in which our experimental animals were kept were so far from those of wild-living lizards as to interfere seriously with their allograft responses.

The third possibility is given support, and the first is shown to be unlikely by experiments on the responses of L. viridis spleen cells to mitogen stimulation in vitro and to stimulation by allogeneic lymphocytes in mixed lymphocyte cultures (Worley & Jurd: unpublished). These preliminary experiments, carried out at a higher temperature (35°C), and in culture conditions where endocrine and other physiological influences are largely absent, show that L. viridis lymphocytes respond promptly and in essentially the same way as those of mammals or other reptiles (18, 19).

In the second part of our study, therefore, we maintained a group of animals in a large enclosure in the open. These lizards were exposed to all the changes in environmental conditions experienced in this area, which is a little to the north of the normal range of the species. (L. viridis is a regular breeder in the wild in the Channel Islands and is successfully bred in captivity in Benfleet, South Essex (G. Webster, personal communication) and in Belgium (20)). The outdoor conditions differed in many ways from those indoors: Figs 1a and 1b show temperature records for a week in August 1978 in the two environments. All the other conditions dictated by the climate and weather also differed: timing and availability of rain and dew, and intensity and duration of insolation may be particularly important. Weather records for the area are shown in Table 4. Protected outdoor conditions such as we provided, and a normal winter hibernation have both been shown to be essential for successful breeding of L. viridis in captivity (G. Webster, personal communication). The highly significant increase in skin allograft rejection in the 'outdoor' group of lizards compared with the 'indoor' animals leads us to suggest that the influence of environmental conditions on immune responses could be of fundamental

relevance in experiments which involve wild-caught animals kept in captivity for extended periods. This assumes increasing importance as comparative immunologists study more and more non-laboratory bred animals.

Many features of the active rejection response of the 'outdoor' group of lizards were not examined because we were unable to obtain a continued supply of animals. In particular, it is necessary to know the histological appearance of the tissues at all stages during rejection, and also to apply a reproducible test of the progress of the allograft-induced cell-mediated immune response in this species, such as the spleen-cell migration inhibition assay recently developed by Jayaraman & Muthukkaruppan (21). Unfortunately it is now not possible to repeat or extend this work in the U.K., so we hope that a European immunology group with better access to L. viridis will be encouraged to take up the study.

In conclusion, we note that our results are in agreement with the recently published report by Borysenko & Lewis on the influence of certain environmental conditions on immunocompetence and resistance to infection in Chelydra serpentina (22). These authors suggest that the cause of poor immune responses in snapping turtles in the laboratory may be malnutrition, and "absence of disease, healthy general appearance and even good appetite, may not be assurance of a species' healthy immunological status". We endorse their views and further suggest that in wholly terrestrial reptiles a number of environmental factors (such as food, insolation, humidity and temperature range) may profoundly influence the animals' immune responses.

TABLE 1
 REPORTS OF SKIN GRAFTING EXPERIMENTS IN SQUAMATES.

Species & Origin	Temperature	Graft Area	Rejection Criteria	Survival Times (days)	Ref.
<u>Anolis carolinensis</u> (Louisiana, U.S.A.)	23.5°C	c. 10-65 mm ²	graft loss	49-90 (allografts)	12
<u>Cnemidophorus sexlineatus</u> <u>viridis</u>	n.a.	c. 25 mm ²	grey graft edges; graft raised above skin; sloughing	13-51 (allografts)	8
<u>C. tessellatus</u> (parthenogenetic)	n.a.	c. 25 mm ²	do.	no rejection	8
<u>C. neomexicanus</u> / <u>C. tessellatus</u>	n.a.	c. 25 mm ²	do.	5-15 (xenografts)	8
<u>Ctenosaura pectinata</u> (Mexico)	25±1°C	c. 100 mm ²	melanophore loss; detachment of graft on palpation	37-87 (1st set allografts)	13
<u>Calotes versicolor</u> (S. India)	28±2°C	c. 225 mm ²	melanophore breakdown; sloughing	24-86 (2nd set)	13
<u>Lepidodactylus lugubris</u> / <u>Hemidactylus turcicus</u> (Hawaii and Louisiana, U.S.A.)	n.a.	c. 6.3 mm ²	graft loss	9-45 (allografts)	3
<u>H. garnotii</u> / <u>H. brookii</u>	n.a.	n.a.	centripetal replacement by host tissues	12-20 (xenografts)	11
<u>Cnemidophorus tigris</u> (Utah, U.S.A.)	36-39°C (light) 21°C (dark)	c. 5 mm ²	sloughing, replacement, melanin accumulation	42-92 (xenografts)	14
<u>Tamnophis sirtalis</u> Manitoba, (Can.)	25±1°C	c. 35 mm ²	n.a.	15-90 'abrupt' 100-350 'gradual' (allografts) to 40 (allografts)	9

(n.a. = data not available)

TABLE 2
REJECTION OF ALLOGRAFTS BY L. VIRIDIS: RESPONSES OF 'INDOOR' LIZARDS (see text)

Group*	No. of animals	No. of allografts per lizard	Grafts unsatisfactory at operation and subsequently ignored	Grafts showing initial signs of rejection: inflammation and/or centripetal colour loss.	Grafts showing total rejection (detachable graft)	Time to 1st signs (days)	Time to total rejection (days)
I	6	1	1	2	1	36-39	c.320
II	28	2	8	1	1	29	c.220
III	4	3	1	1	0	52	---
IV	6	5	2	2	0	28-35	---
V	4	2	0	2(?)	0	14	---
VI	4	4	1	2	0	14	---
TOTAL	52	128	13	10	2	14-39	up to 320

* Group I grafts were performed in February 1977; Groups II, V and VI in June 1977, Groups III and IV in October, 1977. Although L. viridis is a hibernating species of temperate latitudes, the experimental animals were kept at approximately constant temperature in the laboratory (see text) without hibernation.

TABLE 3

REJECTION OF ALLOGRAFTS BY L. VIRIDIS:

SUMMARY OF RESPONSES OF 'INDOOR' AND 'OUTDOOR' LIZARDS (See text)

Group	No. of animals	Total No. of allografts	Grafts ignored because unsatisfactory at operation	Grafts showing initial signs of rejection (inflammation and/or centripetal colour loss)	Grafts showing total rejection	Time to initial signs (days)	Time to total rejection (days)
1	52	128	13	10	2	14-52	220-320
2	6	6	0	1(?)	0	40	No rejection
3	13	26	2	24	24	14-21	32-64

Group 1 is the total of the 'indoor' lizards shown in Table 2.

Group 2 is the 'indoor' control group for Group 3.

Group 3 is the group kept outside (see text).

N.B. Group 2 and 3 animals were supplied in the same batch.

TABLE 4

Weather records (recording station close to the laboratory) for the period August 21st to 28th 1978.

Day	Rain (mm)	Relative humidity*		Temperature °C		Sun
		Day min.	Night max.	Night min.	Day max.	
21 Aug	0	49	97	11.2	23.3	Daily mean 4.82 hours
22	2.4	67	100	14.0	22.8	
23	0	60	100	10.5	18.7	
24	0	56	96	10.1	17.6	
25	0	53	97	8.7	19.2	
26	0	53	96	7.9	17.0	
27	0	58	97	5.7	17.2	
28	0	60	86	10.7	19.0	

* N.B. R.H. inside the laboratory ranged from 45 to 65

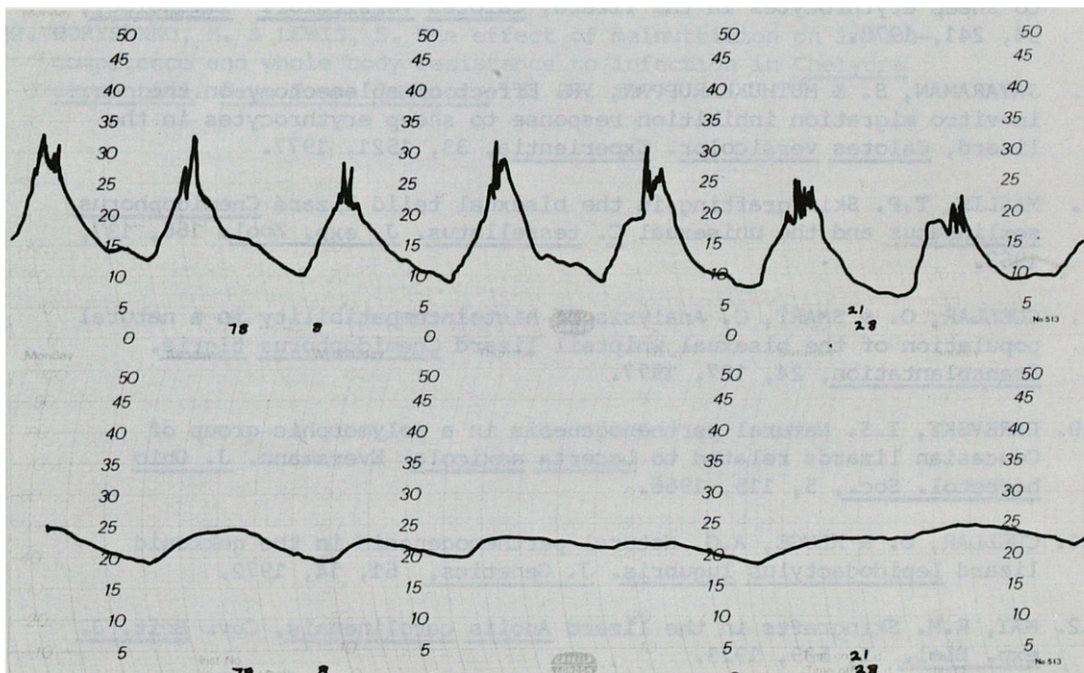


FIG. 1 (a) and (b)

Temperature. (a) outside (upper) and (b) inside (lower) environments.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council (UK). We are grateful to Professor VR. Muthukkaruppan and Dr. RM. Pitchappan for helpful discussion of this paper.

REFERENCES

1. COHEN, N. Reptiles as models in the study of immunity and its phylogenesis. J. Amer. Vet. Med. Assoc. 159, 1662, 1971.
2. BELLAIRS, A. d'A. & ATTRIDGE, J. Reptiles, 4th Edition, p.161. London, Hutchinson, 1975.
3. MANICKAVEL, V. & MUTHUKKARUPPAN, VR. Allograft rejection in the lizard Calotes versicolor. Transplantation, 8, 307, 1969.
4. PITCHAPPAN, RM. & MUTHUKKARUPPAN, VR. Thymus-dependent lymphoid regions in the spleen of the lizard Calotes versicolor. J. exp. Zool. 199, 177, 1977.
5. PILLAI, P.S. & MUTHUKKARUPPAN, VR. The kinetics of rosette-forming cell response against sheep erythrocytes in the lizard. J. exp. Zool. 199, 97, 1977.
6. JAYARAMAN, S. & MUTHUKKARUPPAN, VR. Influence of route and dose of antigen on the migration inhibition and plaque-forming cell responses to sheep erythrocytes in the lizard, Calotes versicolor. Immunology, 34, 241, 1978.
7. JAYARAMAN, S. & MUTHUKKARUPPAN, VR. Effect of splenectomy on the in-vitro migration inhibition response to sheep erythrocytes in the lizard, Calotes versicolor. Experientia, 33, 1521, 1977.
8. MASLIN, T.P. Skin grafting in the bisexual teiid lizard Cnemidophorus sexlineatus and the unisexual C. tessellatus. J. exp. Zool. 166, 137, 1967.
9. CUELLAR, O. & SMART, C. Analysis of histoincompatibility in a natural population of the bisexual whiptail lizard Cnemidophorus tigris. Transplantation, 24, 127, 1977.
10. DAREVSKY, I.S. Natural parthenogenesis in a polymorphic group of Caucasian lizards related to Lacerta saxicola Eversmann. J. Ohio herpetol. Soc., 5, 115, 1966.
11. CUELLAR, O. & KLUGE, A.G. Natural parthenogenesis in the gekkonid lizard Lepidodactylus lugubris. J. Genetics, 61, 14, 1972.
12. MAY, R.M. Skingrafts in the lizard Anolis carolinensis, Cuv. Brit. J. exp. Biol. 1, 539, 1923.
13. COOPER, E.L. & APONTE, A. Chronic allograft rejection in the iguana Ctenosaura pectinata. Proc. Soc. exp. Biol. Med. 128, 150, 1968.

14. ECKARDT, M.J. & WHIMSTER, I.W. Skin heterograft rejection in Hemidactylus garnotii. Copeia, 2, 339, 1973.
15. BORYSENKO, M. Transplantation immunity in reptilia. Transpl. Proc. II, 299, 1970.
16. TEREBEY, N. A light microscopic study of the mononuclear cells infiltrating skin homografts in the garter snake Thamnophis sirtalis (Reptilia: Colubridae). J. Morph. 137, 149, 1972.
17. ARNOLD, E.N. & BURTON, J.A. A Field Guide to the Reptiles and Amphibians of Britain and Europe. London, Collins, (1978).
18. GREAVES, M.F. OWEN, J.J.T. & RAFF, M.C. T and B Lymphocytes: Origins Properties and Roles in Immune Responses. Amsterdam, Excerpta Medica, (1973).
19. CUCHENS, M. McLEAN, E. & CLEM, L.W. Lymphocyte heterogeneity in fish and reptiles. In: Phylogeny of Thymus and Bone Marrow - Bursa Cells. R.K. Wright & E.L. Cooper, (eds), p. 205. Amsterdam: Elsevier/North Holland Biomedical, (1976).
20. SOUWEINE, J. Reproduction in green lizards Lacerta viridis, (L.) in captivity. Nat. Belg. 58, 179, 1977.
21. JAYARAMAN, S. & MUTHUKKARUPPAN, VR. In vitro correlate of transplantation immunity: spleen cell migration inhibition in the lizard, Calotes versicolor. Dev. Comp. Immunol. 1, 133, 1977.
22. BORYSENKO, M. & LEWIS, S. The effect of malnutrition on immunocompetence and whole body resistance to infection in Chelydra serpentina. Dev. Comp. Immunol. 3, 89, 1979.