down feather bulbi model[5]. Morphological analysis showed marked differences in the outgrowth and in the organization of the inner structure of the bulbi.

In the bulbi treated with K, we found a broad margin of outgrowing cells. Hardly any outgrowth could be seen in the serotonin-treated bulbi.

In the K-treated bulbi, the pulpa region is reduced in comparison with control sections. On the other hand, we found several layers of cells in which the appearance of the cellular ultrastructure was normal, but with only minimal signs of keratinization. In the S-treated bulbi, the cells, very limited in number, contained hardly any cell organelles. Meanwhile, keratin accumulation was enhanced.

The tritium activity of the K-treated bulbi was significantly higher than that of the C- and S-treated bulbi, especially after a longer culture period. The higher incorporation of ^3H thymidine reflected a stimulation of the DNA synthesis, as the medium used in the rinsing procedure hardly showed any tritium activity. We can deduce that K directly stimulated the DNA synthesis in epithelial cells, as the majority of cells in our model were keratinocytes[5].

When we combine these findings with the results of previous experiments[3,4], we can conclude that K probably stimulates DNA synthesis and inhibits keratinization in organotypic skin cultures with a majority of fibroblasts, as well as in the precultured down feather bulbi model, with a majority of keratinocytes.

These findings suggest that the wound-healing properties of K can be attributed, at least partially, to a stimulating effect on both dermal and epidermal cells.

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1 International Meeting of Wound Healing Effects of Ketanserin, Janssen Pharmaceutica, Beerse, Belgium, 2 Nov. 1987.
2 Janssen, P. A. J., Janssen, H., Cauwenbergh, G., De Doncker, P., De Beule, K., Tijtgat, H. F., and Lapière, C. M., J. Am. Acad. Dermat. *21* (1989) 85.
3 de Ridder, L., and Beele, H., Experientia *44* (1988) 603.
4 Beele, H., Thierens, H., and de Ridder, L., In Vitro: Cell. devl Biol. *25* (1989) 923.
5 Beele, H., Verhasselt, B., Thierens, H., and de Ridder, L., Experientia *46* (1990) 1053.
6 Jessen, K. R., in: Immunocytochemistry: Practical Applications in Pathology and Biology, p. 169. Eds J. M. Polak and S. Van Noorden. Wright PSG, London 1983.
7 Gilman, R., and Penn, J., Med. Proc. *2* (1956) 93.
8 Vanhoutte, P. M., Prog. appl. Microcirc. *10* (1986) 1.

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In vitro morphological characterization of the bursal reticuloepithelial (REp) cells of chicken

F. Giannessi, F. Bianchi, A. Dolfi, M. Lupetti and S. Michelucci

Chair of Histology and Embryology, Institute of Anatomy, University of Pisa, Via Roma 55, I-56 126 Pisa (Italy)
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Summary. The REp cells of the bursa follicle medulla of chicken were isolated in vitro. Culture of the REp cells was maintained over a period of 10 days and the cells were observed at 3 and 10 days by means of transmission electron microscopy (TEM) and immunofluorescence. The use of an anticytokeratin monoclonal antibody confirmed their epithelial nature. TEM observations showed the presence of desmosomes and tonofilaments, which are characteristic of epithelial cells. Furthermore, to some extent the cells regenerated in vitro the network they form in vivo. Though the growth rate becomes slower with time, the features of the REp cells do not significantly change.
Key words. Bursa of Fabricius; cell culture; cytokeratin; REp cells; electron microscopy.

The Bursa of Fabricius, which is present only in birds, is made up mainly of lymphoid tissue. It is commonly considered to be the primary lymphoid organ for B lymphocyte differentiation. However, in spite of the widespread acceptance of its role in B lymphocyte maturation, the hypothetical factors involved in this maturation are not fully understood. A differentiative microenvironment is thought to promote the maturation of bursal stem cells towards mature B lymphocytes; this point of view has long since been accepted[1-6]. The REp cells of the Bursa of Fabricius are involved in forming the pattern of lymphoid follicle medulla. They are star-shaped when observed in vivo and are linked to one another by processes connected by desmosomes[7-9]. They give rise to a three-dimensional network whose meshes contain medullary lymphocytes[10]. The REp cells possess numerous filaments which react to an anticytokeratin monoclonal antibody[10]. They have an oval nucleus, which is sometimes indented, and often a nucleolus may be clearly observed inside it. The cytoplasm contains small vacuoles, a number of mitochondria and free ribosomes and a few vesicles of the endoplasmic reticulum.

The REp cells are one of the many factors involved in B lymphocyte differentiation in the bursal microenvironment[11,12]. Indeed, the REp cells were observed to promote differentiation into B lymphocytes in 20% of immature lymphocytes, when allowed to grow together with them. We thought it worthwhile to carry out the isolation of the REp cells with a somewhat different technique from that described by Boyd et al.[11], and we made a morphological study for the in vitro description of the cytological and ultrastructural features of REp cells and their changes during culture. The aim of this work is to regenerate in vitro the three-dimensional network made up of REp cells inside which B lymphoid cells are collected in the organ, and thus to assess the basic morphofunctional conditions of a model suitable for further experiments. This model should throw light on the interrelationships between REp cells and B lymphocytes, and the possible factors involved in differentiation. For this reason REp cells were cultured for 10 days and observed by means of TEM, and with immunofluorescence after treatment with an anticytokeratin monoclonal antibody.

Materials and methods

The experiment was carried out on bursae from 8-week-old chicks of the Red and Grey strain, hatched from eggs obtained from a local hatchery and incubated in our laboratory in the usual way. The animals received food and water ad libitum until the experiment was started. The chicks were killed by cervical dislocation and their bursae were removed with sterile methods and instruments. After several washings in sterile PBS, the bursae were transferred to a RPMI 1640 medium without FCS, and were dissociated by gently squeezing with forceps. In this way, the follicle medullae were taken out of the bursal follicles in which they are normally located[11], and a suspension was obtained containing lymphocytes and fragments of the bursal follicle medullae. After the medullae had sedimented, the supernatant with free lymphocytes was discarded; then the remaining medullae were shaken several times, to free the medullar lymphocytes from the medullae. Then the fragments were sedimented again and the supernatant discarded. This was repeated until the supernatant was clear.

The fragments were then resuspended in RPMI 1640 medium with 10% FCS, seeded in 1-ml well plates and incubated in an incubator in 37 °C in a 5% CO_2 atmosphere. After 12–24 h the medium was discarded and the fragments were supplied with fresh medium. After the fragments had generated monolayers, the supernatant, where clustered cells were found to be growing, was collected and placed in 25-ml flasks with the same medium to which 10% FCS had been added. The culture was maintained over a period of 10 days, by changing the medium every 3 days. The cells were collected at 3 and 10 days to carry out morphological studies. For immunofluorescence, the cells were stratified on a cover slip, stuck on and air-dried; then the cells were fixed for 10 min with a mixture of 6 parts of ethanol, 3 of chloroform, and 1 of acetic acid.

Immunofluorescence was carried out with the anticytokeratin monoclonal antibody we had already used[10] as the first antibody, and with a fluoresceine-linked antibody as the second antibody. The process was carried out in accordance with the standard methods. Peripheral blood cells were used as negative controls. In particular, the REp cells were prepared for TEM by centrifugation at 400 × g for 10 min in Falcon U-bottom test-tubes; glutharaldehyde 1% – paraformaldehyde 4% was then used as a fixative for 2 h; post-fixation in 1% osmium tetroxide was performed for 1 h, and lastly the cells were embedded in Epon after dehydration in a graded series of alcohols. Ultrathin sections were cut with an Ultrotome Nova LKB (Sweden) ultramicrotome and were stained with uranyl acetate and lead citrate before observation with a Siemens Elmiskope 101 transmission electron microscope.

Results

The cultures were observed by means of a Leitz Diavert inverted phase contrast microscope. At the beginning,

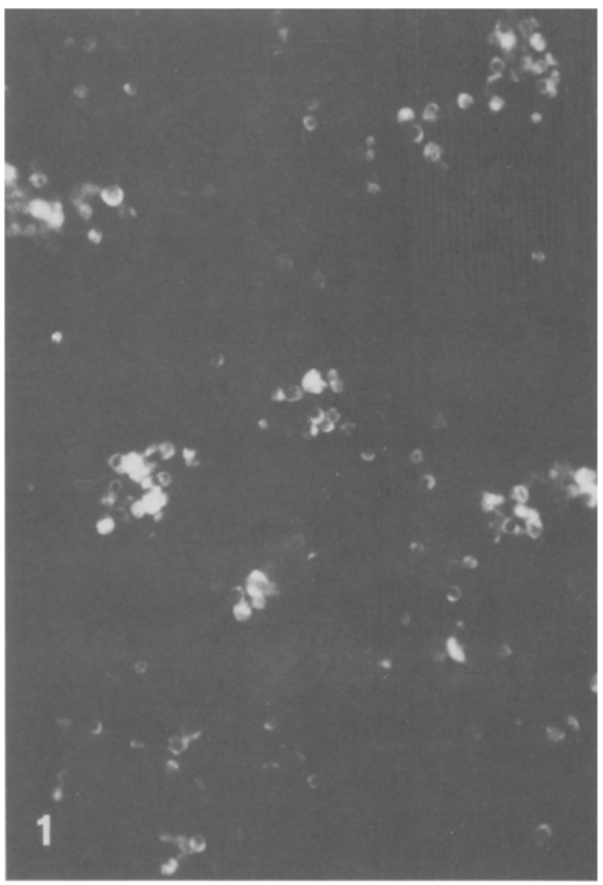
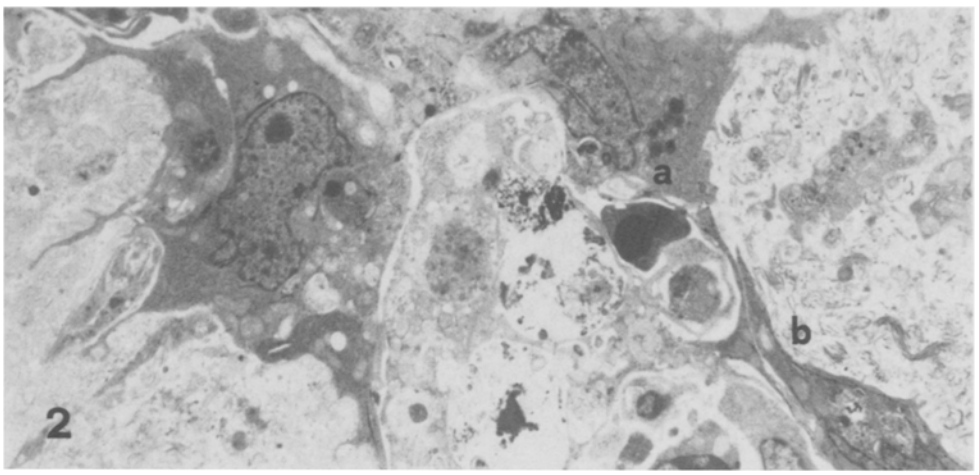
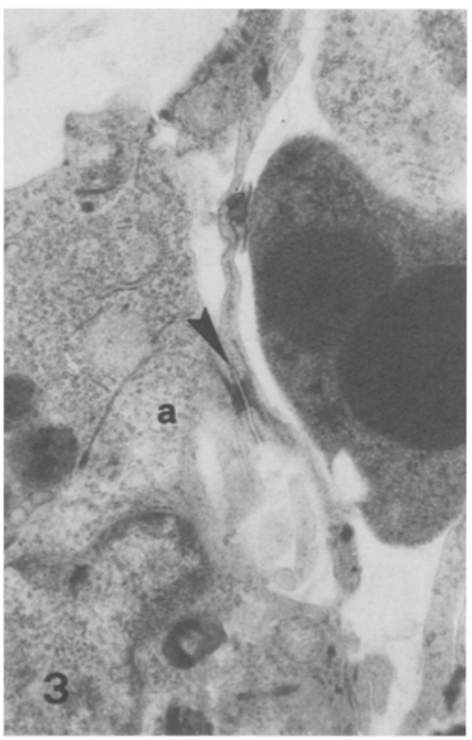


Figure 1. Anticytokeratin monoclonal antibody immunofluorescence on isolated REp cells. One kind of cell alone is present; this shows variable degrees of positivity. Some of the cells give rise to small clusters. Reactivity is present in the cytoplasms.

they contained various cellular types, including the residual lymphocytes. Then the fragments started an eccentric proliferation, giving rise to two kinds of cells: some of them adhered to the plastic, forming a monolayer, while the remaining cells grew in suspension and formed clusters of variable numbers of cells. These seemed to be REp cells. While the culture liquid was being changed, the supernatants were collected and placed in 25-ml flasks for further cultivation, fluorescence and TEM studies. The cellular population we obtained appeared to contain the clustering cells alone. These cells grew in log phase for the first week after the explant, but growth became slower during the last days of observation. The adhering cells resembled phagocytic and fibroblastic cells, and now and then hemopoietic cells formed colonies.

Fluorescence observation showed that the cultured REp cells did indeed continue to be strongly reactive to an anticytokeratin monoclonal antibody. The pattern shown in figure 1 is that of cells three days after explant, but it does not significantly differ from that encountered in cells which were cultured for longer periods: in particular, REp cells show a positivity which is widely spread over all their cytoplasm in the large majority of cases. Many cells grow to form small clusters. The peripheral





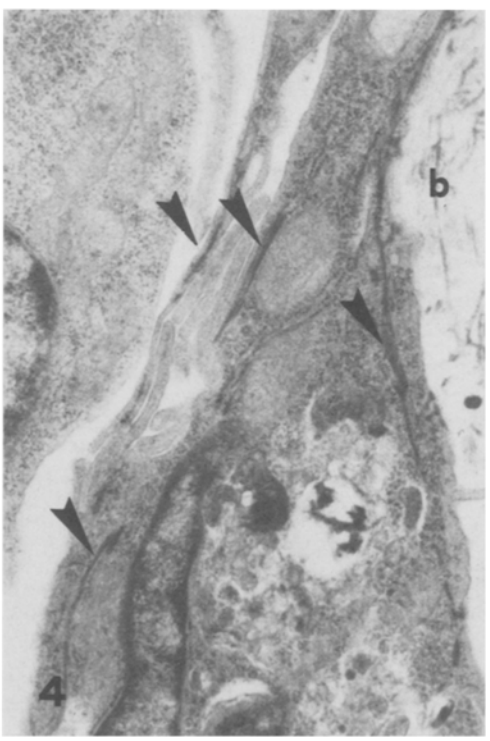


Figure 2. REp cells cultured in vitro. TEM observation. The pattern of the REp cells is typical, they contain a large irregular nucleus with a nucleolus, and numerous cytoplasmic processes. The cells recreate a three-dimensional network in vitro by connecting their processes. Dead cell debris is present inside the meshes of this network. × 4000

Figure 3. Magnification of figure 2. A desmosome connecting the processes belonging to two REp cells can be seen. × 19.500

Figure 4. Numerous filaments are present inside the REp cells and their processes. × 19,500

blood lymphocytes we used as controls proved to be totally negative.

TEM, at small magnifications, showed cells with a large irregular nucleus containing a regular nucleolus. The cells were connected by processes forming a three-dimensional network (fig. 2). A few lymphoid cells and other degenerating cells were observed inside the meshes of this network during the previous stages of culture. At a later stage, flocculent material and dead cell debris appeared among the meshes (fig. 2). Certain cytological features were observed at greater magnifications. In 3-day cultures, free ribosomes were prominent, and vacuoles were present, together with filaments presumably belonging to the class of keratin-made tonofilaments (fig. 3). Desmosomes connecting the cellular processes to one another were frequently observed (fig. 4).

Discussion

The aim of the present work was to isolate REp cells from the medulla of chicken bursal follicles, and to characterize them by means of immunofluorescence with an anticytokeratin monoclonal antibody and TEM. Our results show that the method we used makes it possible to isolate REp cells. This is demonstrated by the positivity towards the anticytokeratin monoclonal antibody and by the ultrastructural features of the cultured cells. Indeed, the immunofluorescence technique shows an intense positivity in all the cells, while electron microscopy shows the typical morphological pattern of the REp cells; desmosomes, connecting the cellular processes to one another, and tonofilaments were the main features. REp cells do not seem to modify their pattern during the period of culture, even if growth becomes slower after a log phase during the first week of culture. In the past, a similar method was used by others to isolate the cells of the bursal follicle medullae. Boyd et al. used virtually the same technique of dissociation, followed either by irradiation [11], or enzymatic treatment [9], to improve the isolation of the REp cells and to eliminate lymphocytes. In the present work, we show that successive changes of the medium make it possible to eliminate lymphocytes. The adhering non-epithelial cells are discarded because the REp cells stick on lightly, and gentle shaking resuspends them, so that it is then possible to seed supernatants that contain REp cells. The possibility of isolating REp cells, and regenerating in vitro the three-dimensional network that they normally form in vivo, may be an interesting starting-point for further morphological and biochemical research on interactions between bursal lymphocytes and epithelial cells during lymphocyte differentiation in the primary lymphoid organs.

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1 Toivanen, P., Toivanen, A., and Good, R. A., J. Immun. *109* (1972) 1058.
2 Boyd, R. L., Ward, H. A., and Muller, H. K., Int. Arch. Allergy appl. Immun. *50* (1976) 129.
3 Lydyard, P. M., Grossi, C. E., and Cooper, M. D., J. exp. Med. *144* (1976) 79.
4 Glick, B., and Olah, I., Immun. Today *5* (1984) 152.
5 Glick, B., and Olah, I., in: Avian Immunology, p. 53. Eds W. T. Weber and D. L. Ewert. Alan R. Liss Inc., New York 1987.
6 Sorrell, J. M., Lintala, A. M., Mahmoodian, F., and Caterson, B., J. Immun. *140* (1988) 4263.
7 Frazier, J. A., Acta anat. *88* (1974) 385.
8 Naukkarinen, A., and Sorvari, T. E., Acta path. microbiol. scand. (C) *90* (1982) 193.
9 Boyd, R. L., Mitrangas, K., Ramm, H. C., Wilson, T. J., Fahey, K. J., and Ward, H. A., in: Avian Immunology, p. 41. Eds W. T. Weber and D. L. Ewert. Alan R. Liss Inc., New York 1987.
10 Lupetti, M., Dolfi, A., Giannessi, F., Bianchi, F., and Michelucci, S., Am. J. Anat. *187* (1990) 287.
11 Boyd, R. L., Ward, H. A., and Muller, H. K., J. Reticuloendoth. Soc. *34* (1983a) 371.
12 Boyd, R. L., Ward, H. A., and Muller, H. K., J. Reticuloendoth. Soc. *34* (1983b) 383.

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Novel oscillations in cell suspensions of *Dictyostelium discoideum*

B. Wurster

Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz (Federal Republic of Germany)
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Summary. With a light-scattering technique, two novel rhythms were discovered in cell suspensions of *Dictyostelium discoideum*. One is a damped oscillation with a period of 2 to 2.5 min (at 23 °C) induced by folate in EDTA-dissociated undifferentiated cells. The other is a sinusoidal oscillation with a period of about 12 min occasionally observed with late differentiated cells. Obviously, the repertoire of rhythms of this simple eukaryotic organism is larger than previously assumed.
Key words. Dictyostelium; cell communication; biological rhythms; oscillations; cAMP; folate; calcium.