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Volume 108

Immunochemical Techniques

Part G
Separation and Characterization of Lymphoid Cells

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IMMUNOCHEMICAL TECHNIQUES

Part G

Separation and Characterization of Lymphoid Cells

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Section I

Surgical Techniques in Immunology

LL-1014

[1] Methods of Bursectomy

By BRUCE GLICK and IMRE OLÁH

The bursa of Fabricius is a dorsal diverticulum of the proctadael region of the cloaca.[1,2] A fortunate observation elevated the bursa to a prominent position.[3-5] Bursectomy was shown to markedly reduce or eliminate humoral immunity.[5,6] The historical and recent experiments with the bursa have been reviewed.[2,7] This chapter will critique the various methods of bursectomy.

Surgical Bursectomy: Posthatch

A variety of anesthetics have been tested in the neonatal chicken.[8] We routinely administer sodium thiopental (Dipentol, Diamond Laboratories) intraperitoneally (ip) or intravenously (iv) to newly hatched chicks or iv at later ages. Newly hatched chicks will tolerate between 0.025 and 0.1 ml of sodium thiopental (30 mg/ml) when administered ip. Administering the lower volume allows one to determine the degree of sensitivity of each group of chicks. The chicks are anesthetized within 3 min of the ip injection. Intravenous injection of sodium thiopental should be at the level of 1 mg/30 g of body weight with a 20-sec interval separating each 0.1 ml injected. Anesthesia immediately follows the iv injection.

With the thumb and forefinger of the left hand the tail is raised to a vertical position, the few feathers beneath the tail are removed, and the area is swabbed with 70% ethanol (EA) (Fig. 1). A superficial-lateral incision, 3–6 mm, at the base of the tail and just above the upper lip of the vent is made.[2-5] A minimum of bleeding is experienced. Too large an incision may allow the yolk sac to enter the orifice. Curved forceps must be used to break through the remaining connective tissue and enter the body cavity. Gentle pressure with the left hand, applied to the dorsal

[1] J. Jolly, *Arch. Anat. Microsc. Morphol. Exp.* **16,** 363 (1915).
[2] B. Glick, *in* "Avian Biology" (D. S. Farner, J. F. King, and K. C. Parkes, eds.), Vol. 7, pp. 443–500. Academic Press, New York, 1983.
[3] B. Glick, Ph.D. Dissertation, Ohio State University, Columbus (1955).
[4] T. S. Chang, B. Glick, and A. R. Winter, *Poult. Sci.* **34,** 1187 (1955).
[5] B. Glick, T. S. Chang, and R. G. Jaap, *Poult. Sci.* **35,** 224 (1956).
[6] M. D. Cooper, R. D. A. Peterson, M. A. South, and R. A. Good, *J. Exp. Med.* **123,** 75 (1966).
[7] B. Glick, *BioScience* **33,** 187 (1983).
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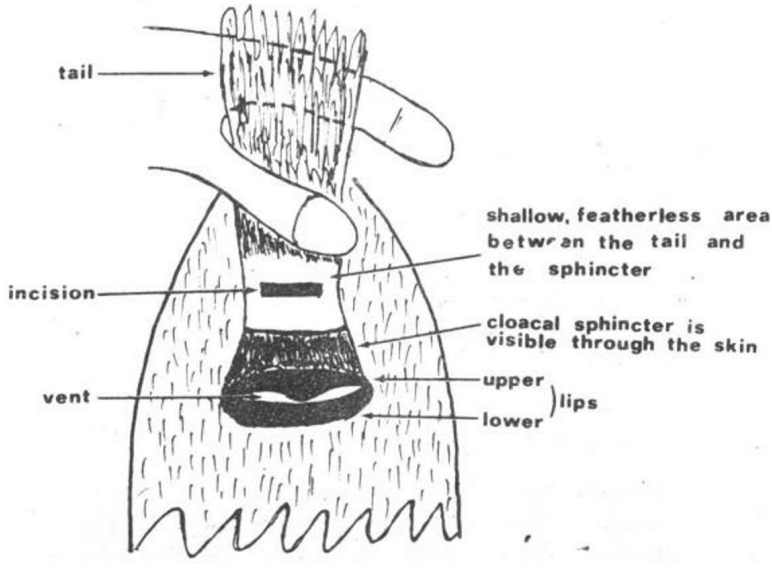

FIG. 1. Posterior aspect. Location of incision with reference to tail and upper lip of vent.

surface of the chick, will force the apex of the bursa into the newly made orifice (Fig. 1). The exposed bursa is grasped with curved forceps and is extended beyond the orifice until the bursal duct is viewed. The duct is severed at its point of attachment with the cloaca and large intestine (Fig. 1). The incision may be sutured; however, without sutures the incision will completely heal within 1 week. The bursa must be removed intact since damage to its serosa could lead to the release of bursal lymphocytes into the body cavity. Lymphoid cells may be identified along the bursal duct (Fig. 2). Therefore, it is important to sever the duct flush with its attachment to the alimentary canal. Additional lymphoid cells, apparently T cells,[9] are observed dorsal to the duct. Surgical bursectomy performed at hatch is more effective than bursectomy performed at later ages.[10-12] However, numerous investigators reported that chicks bursectomized at hatch responded to a secondary and tertiary injection of antigen.[13] Enhanced immunosuppression occurs at hatch when bursectomy is followed within 24 hr by irradiation (6 R).[6]

[9] S. Odend'hal and J. E. Breazile, *J. Reticuloendothel. Soc.* **25,** 315 (1979).
[10] T. S. Chang, M. S. Rheins, and A. R. Winter, *Poult. Sci.* **36,** 735 (1957).
[11] A. P. Mueller, H. R. Wolfe, and R. K. Meyer, *J. Immunol.* **85,** 172 (1960).
[12] M. A. Graetzer, W. P. Cote, and H. R. Wolfe, *J. Immunol.* **91,** 576 (1963).
[13] B. Glick, *Int. Rev. Cytol.* **48,** 345 (1977).

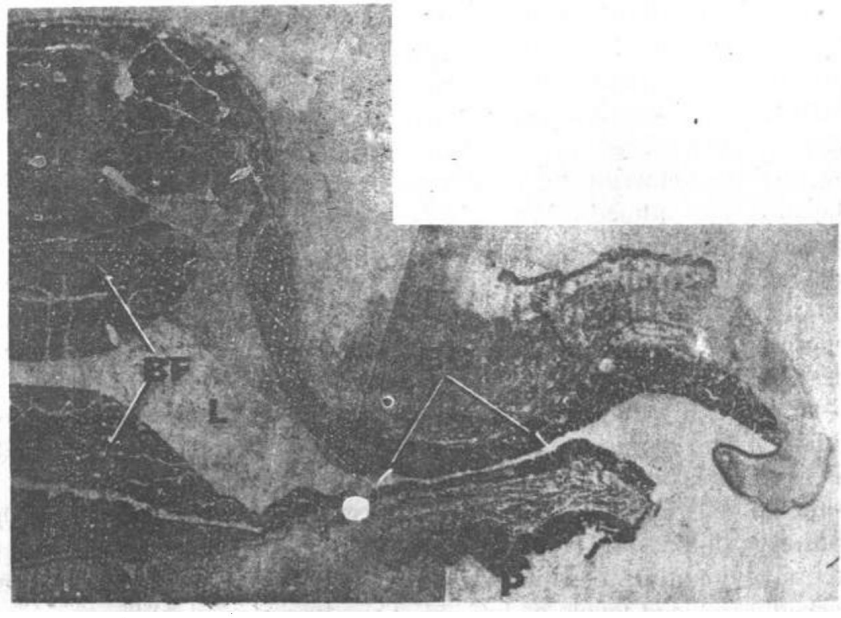

FIG. 2. Lymphocytes collect along the bursal duct (BD) which leads into the bursa's lumen (L). Bursal follicles (BF). Lymphoid tissue in the proctacdael area (P). Magnification: ×7.

Hormonal Bursectomy

With the observation that androgen injections of neonates produced bursa regression, Meyer *et al.*[14] administered 19-nortestosterone *in ovo* and arrested the lymphoid development of the bursa. In his laboratory a variety of androgens were evaluated. It was found that 0.5 mg of androsterone or 1.5 mg of androstane-3,17-dione or 1.5 mg of dehydroepiandrosterone administered on the fifth day of embryonic development produces bursaless embryos.[15] On the fifth day of embryonic developoment, a hole is made at the pointed end of the egg with a 23-gauge needle. With a 20-gauge needle, 19-nortestosterone (0.63 mg in 0.1 ml corn oil) is injected into the albumin. The hole is sealed with paraffin or transparent tape. Precipitin response to bovine serum albumin is eliminated in chicks hatched from these eggs while delaying the injection of 19-nortestosterone

[14] R. K. Meyer, Rao M. Appaswamy, and R. L. Aspinall, *Endocrinology (Baltimore)* **64**, 890 (1959).

[15] R. L. Aspinall, R. K. Meyer, and M. A. Rao, *Endocrinology (Baltimore)* **68**, 944 (1961).

until the twelfth day of incubation fails to eliminate the precipitin response.[11,16] The injection of 2.5–4.0 mg of testosterone propionate (TP) into the allantoic cavity on the eleventh or twelfth day of embryonic development prevents the appearance of lymphoid tissue in the bursa of Fabricius.[17] The egg is candled (passage of an incandescent wavelength of light through the egg) to reveal the air cell, which is in the large end of the egg, and its semipermeable membrane below which develops the embryo. The air cell is outlined with a pencil. Then, an area about 3 mm below the semipermeable membrane is wiped with 70% EA and pierced with a 23-gauge needle. Testosterone propionate (50 mg/ml, 0.05–0.1 ml) dissolved in autoclaved sesame oil is injected into the allantoic cavity. The hole is covered with paraffin or transparent tape. Hatchability of the injected eggs will be at least 50%.

Hormonal bursectomy may be effected by dipping eggs in steroid solutions.[18,19] The dipping technique will either eliminate the bursa in the hatched chick or markedly reduce its size with general hypertrophy of epithelial cells and inhibition of bursal lymphopoiesis.[20] Chicks hatched from eggs dipped in TP, unlike their TP-injected counterparts, produce excessive quantities of immunoglobulin M (IgM), the pentameric Ig, and markedly reduced levels of IgG.[21,22] This dysgammaglobulinemic model allowed us to conclude that, at least, IgM synthesis has a bursal independent pathway.[21–23] The pointed end (2–4 cm) of a 3-day-old embryonated egg is dipped for 5 sec into an EA-TP solution and then returned to the setting tray. The EA-TP solution is maintained at 18°. Concentrations of TP below 1.2 g% (1.2 g TP in 100 ml EA) will have varying effects on bursal development while EA containing 2 g% TP will eliminate the bursa in 80% of the hatched chicks. Bioassay and isotope procedures reveal that the albumin of eggs which are dipped in EA solutions (0.5–0.8 g% in TP) contains between 74 and 111 μg of TP within 60 min of shell exposure.[24,25]

Surgical Bursectomy in Ovo

In ovo bursectomy between 17 and 19 days of embryonic development lead to the proposal that the bursal microenvironment is necessary for the

[16] A. P. Mueller, H. R. Wolfe, R. K. Meyer, and R. L. Aspinall, *J. Immunol.* **88**, 354 (1962).
[17] N. L. Warner and F. M. Burnet, *Aust. J. Biol. Sci.* **14**, 580 (1961).
[18] B. Glick and C. R. Sadler, *Poult. Sci.* **40**, 185 (1960).
[19] B. Glick, *Endocrinology (Baltimore)* **69**, 984 (1961).
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[22] D. S. V. Subba Rao, F. C. McDuffie, and B. Glick, *J. Immunol.* **120**, 783 (1978).
[23] B. Glick, *Proc. Soc. Exp. Biol. Med.* **127**, 1054 (1968).
[24] J. A. Wilson and B. Glick, *J. Miss. Acad. Sci.* **12**, 308 (1966).
[25] J. A. Wilson, N. Mitlin, and B. Glick, *Poult. Sci.* **50**, 655 (1971).

transformation of IgM to IgG.[26,27] Eggs are prepared for *in ovo* bursectomy by candling, wiping the lower half of the shell with 70% EA, and then cutting a 1.5 × 2-cm window in the region covering the caudal portion of the embryo. The caudal portion of the embryo is pulled through a small orifice in the vascular chorioallantoic membrane. The bursa is removed as described in the section on surgical bursectomy. The embryo is returned to the interior and the opening sealed with transparent tape. All the procedures are performed aseptically. Survival should be approximately 70%.

Experiments with monospecific IgM serum (anti-μ-serum) and IgG serum (anti-γ-serum) revealed that IgM appeared in the bursal cells by day 14 of embryonic development, IgG by day 21, and occasional bursal cells possessed IgM and IgG on the twenty-first day of embryonic development.[28] The injection of anti-μ (0.1–0.2 ml of 1.9 mg antibody) into a chorionic vessel (see cyclophosphamide procedure for *in ovo* iv injections) on day 13 of embryonic development followed by bursectomy at hatch will suppress synthesis of IgM and IgG.[29]

A bursal independent pathway for the synthesis of IgM was identified in our laboratory employing hormonal bursectomy.[2,13] Verification and extension of our data have come from *in ovo* bursectomies performed prior to 72 hr of incubation.[30–33] The *in ovo* bursectomies performed at or before the 18 somites stage eliminated the bursa and one-third of the large intestine in hatched chicks, but did not eliminate B cells or IgM synthesis. A paraphrase of Fitzimmons' *in ovo* bursectomy[30] procedure follows.

Eggs are incubated for approximately 3 days and then placed small end up for 1 hr. They are wiped with 70% EA. Albumin and shell membranes are collected from a single egg for later use. An orifice about 1 cm in diameter is cut at the small end of the shell. This will reveal the embryo (Fig. 3a). The embryo is brought to the top of the hole by the addition of albumin. Using a microscope, a small slit is made in the chorion posterior

[26] P. J. Van Alten, W. A. Cain, R. A. Good, and M. D. Cooper, *Nature (London)* **217**, 358 (1968).

[27] M. D. Cooper, W. A. Cain, P. J. Van Alten, and R. A. Good, *Int. Arch. Allergy* **35**, 242 (1969).

[28] P. W. Kincade and M. D. Cooper, *J. Immunol.* **106**, 371 (1971).

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[30] R. C. Fitzimmons, M. F. Garrod, and I. Garnett, *Cell. Immunol.* **9**, 377 (1973).

[31] D. X. Dixon and R. C. Fitzimmons, *Dev. Comp. Immunol.* **4**, 713 (1980).

[32] B. D. Janković, Z. Knezević, K. Isaković, K. Mitrović, B. M. Marković, and M. Rajčević, *Eur. J. Immunol.* **5**, 656 (1975).

[33] B. D. Janković, K. Isaković, B. M. Marković, M. Rajčević, and Z. Knezević, *Exp. Hematol.* **4**, 246 (1976).

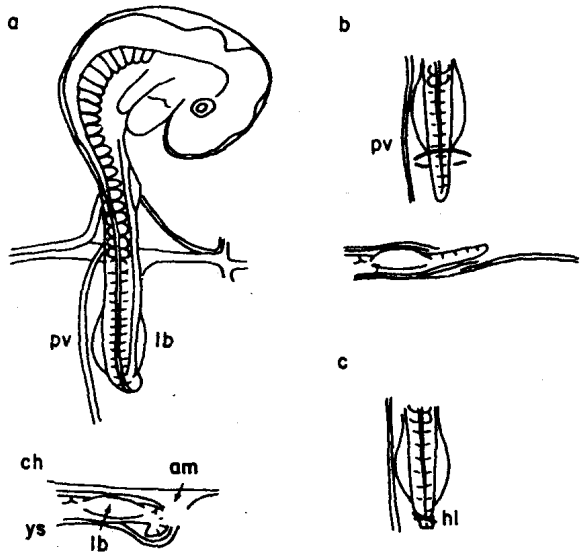

FIG. 3. (a) Embryo (72-hr) with side view of tail area to show extraembryonic membranes. am, Amnion; ch, chorion; lb, leg bud; pv, posterior vitelline vein; ys, yolk sac membrane. (b) Tail region with membranes cut and tail protruding. (c) Tail tied with hair loop and tip amputated. hl, Hair loop. Reproduced with permission of Fitzimmons *et al.*[9]

to the leg bud. The tip of a knife is inserted to one side of the midline and the opening is extended to the opposite side. The vitelline circulation and yolk sac should not be damaged. Cut the amnion and draw out the tip of the tail to the top of the chorion (Fig. 3b). Desiccation is prevented by the addition of albumin or Ringer's solution. A loop of fine hair, previously rinsed in 70% EA, is positioned over the free end of the tail, posterior to the leg buds, and tightened with forceps. The loop will prevent blood flow through the paired dorsal aorta. The ends of the hair are trimmed. The tail posterior to the loop is severed and removed (Fig. 3c). The tail stump is returned beneath the chorion. Following the addition of albumin to fill the air space, an appropriate size of double shell membrane is placed over the opening. In a few minutes, the albumin on the underside of the membrane will dry and assist sealing. The sealing is completed by applying a thin layer of paraffin over the edges of the double shell membrane. The eggs are now ready for normal incubation.

Cyclophosphamide

The ontogeny of humoral immunity is suppressed when chicks are injected on the first 3 days after hatch with 4 or 6 mg of cyclophosphamide

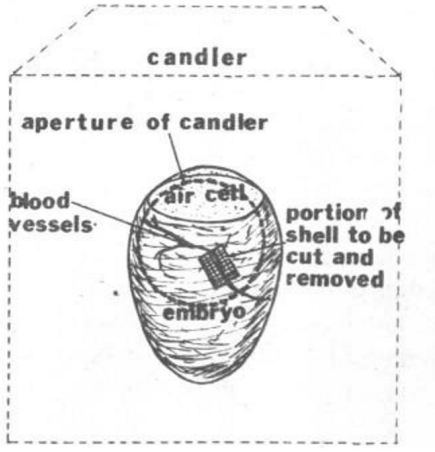

FIG. 4. A fertile egg candled to view location of blood vessels.

(Cy).[34] Cy eliminates lymphocytes from the bursa but not the thymus and magnifies the presence of bursal secretory cells.[35–38] An aqueous solution of 0.1 or 0.2 ml Cy (20 mg/ml) is injected intramuscularly the first 3 days after hatching. Higher doses will lead to excessive mortality during the first 3 weeks of age.[34] The ability of Cy to suppress bursal lymphopoiesis and humoral immunity may depend on the breed or strain of chickens.[39]

Chemical bursectomy with Cy has also been effected with intravenous injections at 12 to 14, 14 to 16, and 16 to 18 days of embryonic development.[40,41] The greatest mortality occurs in the youngest embryos with the most effective humoral immune suppression observed in the 16- to 18-day group. Eggs are candled at 16 days of embryonic development and a major vein is outlined in pencil (Fig. 4). The area is disinfected with 70% EA. A variable drill is used to cut the square piece of shell which overlays the vein. The shell window is removed with curved forceps. The exposed membrane is cleared with mineral oil. Cy (0.1 ml, 2 mg or 1 mg/10 g body weight) is injected with a 30-gauge needle (bevel up) attached to a tuberculin syringe. Allow several seconds before removing the needle. Cover

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