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Induction of cell–cell adhesion by monovalent antibodies in a germ cell culture

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SUMMARY

Four monoclonal antibodies, XT-I, MT-23, MT-24 and MT-29, that bind the XT-1-differentiation-antigen of male germ cells have been used to investigate the biological role of the XT-1-molecule of germ cells in short-term primary culture. Cultures from 10 days *postpartum* mice demonstrate increasing numbers of antigen-positive germ cells and increased antigen expression per cell with succeeding days of culture. Treatment of the antigen-positive cultures with three of the monoclonal antibodies, XT-I, MT-23 and MT-24, increases germ cell–germ cell adhesion in a dose-dependent fashion. Treatment with the fourth monoclonal antibody, MT-29, does not induce cell adhesion. The monovalent, Fab fragment of XT-I-antibody also elicits tight cell adhesion, thus ruling out antibody cross linking of molecules or cells. Saturating or near saturating amounts of the positive antibodies are required to produce adhesion, a result consistent with perturbation of a function that is performed by the sum of action of many of the XT-1-molecules on the cell. The ability of germ cells to undergo antibody-elicited tight adhesion is dependent on germ cell age and/or XT-1-antigen concentration. We hypothesize that the XT-1-molecule is involved in regulation of cell adhesion, an event which must occur in normal development.

INTRODUCTION

The ability to study a biological phenomenon *in vitro* is essential to the characterization of the molecular mechanisms involved. Various developmental systems have been fruitfully investigated *in vitro*, including the regulation of cartilage formation (Hall, 1983), and the specificity and mechanisms of cell adhesion (Damsky *et al.* 1983; Edelman, 1983).

One of the least understood developmental systems is that of male germ cells. Studies on regulation of mammalian germ cell differentiation have been complicated by the fact that germ cells become sequestered by the blood–testis barrier, making it difficult to monitor their differentiation and to manipulate their environment. Recently, however, researchers have devised improved culture conditions for continuous culture of Sertoli cell lines and for primary cultures of germ cells along with Sertoli cells, opening this system to *in vitro* analysis. In culture, isolated germ cells are capable of incorporating labelled precursors for

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only several hours (Grootegoed, Grollé-Hey, Rommerts & Van der Molen, 1977; Grootegoed *et al.* 1982; Millette & Moulding, 1981; Silver, Uman, Danska & Garrels, 1983). In contrast, when in contact with Sertoli cells in serum-free culture conditions, spermatogonia and spermatocytes survive and continue to differentiate (Palombi *et al.* 1979; Tres & Kierszenbaum, 1983). The germ cells in these systems have been identified by their light-microscopic (Tres & Kierszenbaum, 1983) and electron-microscopic morphology (Eddy & Kahri, 1976; Palombi *et al.* 1979).

From initial studies (Bechtol, Brown & Kennett, 1979; Bechtol, Jonak & Kennet, 1980) it was apparent that the XT-1-differentiation-antigen could be a useful marker in experimental situations such as *in vitro* culture. In histologic section of juvenile and adult mouse testes, XT-1-antigen is localized by immunoperoxidase staining on the cell surfaces of early spermatocytes (leptotene/zygotene) and later germ cells, while Sertoli cells are antigen-negative (Bechtol, 1984). Detection of the antigen is thus dependent on the presence of germ cells of the appropriate developmental stage, and is a marker of the germ cells and their state of development.

Specific antibodies can affect biological function and provide useful tools for analysis of the molecules and mechanisms involved (Beug *et al.* 1970; Kemler, Babinet, Eisen & Jacob, 1977; Uchiyama, Broder & Waldmann, 1981*a*; Uchiyama, Nelson, Fleisher & Waldmann, 1981*b*; Ware *et al.* 1983). Here we have used four monoclonal antibodies that bind the XT-1-molecule to investigate its biological role on germ cells. In addition, results presented in this study provide biochemical evidence for the development of germ cells *in vitro*. This and the detailed morphological description of germ cells in similar cultures (Eddy & Kahri, 1976; Palombi *et al.* 1979; Tres & Kierszenbaum, 1983), suggest that a germ cell culture system is a particularly appropriate model for studying the molecular mechanisms of germ cell differentiation.

MATERIALS AND METHODS

Animals and reagents

Strain 129/SV mice were maintained as an inbred colony at The Wistar Institute. W^x/W^v, black-eyed white offspring were bred from $W^x/+$(C3H/HeJ) (Russell, Lowson & Schabtach, 1957) crossed with $W^v/+$(C57B1/6J) obtained from Jackson Laboratories. All chemicals unless otherwise stated were from Sigma.

Primary outgrowth cultures from juvenile seminiferous epithelium

Testes from juvenile [10 to 12 days *postpartum* (*p.p.*) or 17 days *p.p.*] mice of strain 129 or W^x/W^v were placed in culture according to the method of Mather & Sato (1979) with slight modifications (Mather & Phillips, 1984). Briefly, testes were decapsulated in warm SF/F12/DMEM (serum-free, 1:1 mix of Ham's F12 and Dulbecco's minimum essential medium, Gibco) and teased with forceps. The seminiferous tubules were dispersed by pipetting in solution A [1 M-glycine, 2 mM-ethylene diaminetetraacetate (EDTA), 0·002 % soybean trypsin inhibitor, and 200 i.u. ml^{-1} DNase I in PBS, pH 7·2] for 5 min, then allowed to settle at unit gravity for 10 min and washed three times with SF/F12/DMEM. The dispersed tubules were minced with

scissors and incubated at 32°C for 30 min each with two successive aliquots of 5 ml solution B (0·05 % collagenase/dispase, Boehringer–Mannheim, 0·005 % soybean trypsin inhibitor and 200 i.u. ml^{-1} DNase I in SF/F12/DMEM). The tubule chunks are then minced again, washed three times with SF/F12/DMEM and resuspended in SF/F12/DMEM plus supplement [5 μg insulin ml^{-1}, 5 μg transferrin ml^{-1}, 30 nM-selenius acid (ITS, Collaborative Research), 3 nM-progesterone, 1 μg epidermal growth $factor^{-1}$ (Collaborative Research), 0·5 ng ovine follicle-stimulating hormone ml^{-1} (Pituitary Hormone Distribution Program, Bethesda, MD), 50 μg retinoic acid ml^{-1}, 100 i.u. penicillin ml^{-1} (Gibco), 100 μg streptomycin ml^{-1} (Gibco), and 4 mM-glutamine (Gibco)]. At this point, the intertubular cells and myoid cells have largely been removed so that the tubule chunks consist almost exclusively of germ cells and Sertoli cells (Mather & Phillips, 1984). The suspension of tubule chunks is centrifuged (800 *g* for 10 min) in a McNaught protein test tube for estimation of total cell number and the pellet is resuspended with SF/F12/DMEM plus supplement to a final concentration of 10^6 cells ml^{-1}. 100 μl aliquots of the tubule suspension are seeded onto individual wells of a 24-well tissue-culture plate (Costar) and maintained in 1·0 ml SF/F12/DMEM plus supplement at 32°C in 5 % CO_2 in air. There were 15 to 25 outgrowths of tubule fragments per well. The great majority of epithelioid cells in the outgrowth cultures are identified as Sertoli cells on the basis of their refractile inclusions, their rapid uptake of India ink, their characteristic nuclear shape in electron microscopy, and their low level expression of H-2. The majority of round, refractile cells overlying the Sertoli cells are identified as germ cells because they express the XT-1 differentiation antigen and in many cases are connected by cytoplasmic bridges.

Monoclonal antibodies

The mouse monoclonal antibody XT-I (IgG_{2a}) was derived as previously described (Bechtol *et al.* 1979) and was produced from a subline secreting only the Ig heavy and light chains of the lymphocyte parent (Bechtol *et al.* 1985). Rat monoclonal antibodies MT-23 (IgG_{2a}), MT-24 (IgG_{2b}) and MT-29 (IgG_{2a}) were produced by immunization with substantially enriched XT-1-antigen, and all immunoprecipitate the XT-1-antigen (Bechtol *et al.* 1985). The anti-H-$2K^b$, D^b mouse hybridoma line HB-11 (IgG_{2a}, Ozato & Sachs, 1981, 20-8-4S) was obtained from the American Type Culture Collection. All antibodies were produced in serum-free culture (Murakami *et al.* 1982).

Indirect immunoperoxidase staining

Cultures were fixed with a 1:1 mixture of Bouin's fixative and phosphate-buffered saline (PBS) for 10 min at room temperature, then rinsed for 5 min with PBS and air dried. If previously untreated with antibody, fixed cultures were incubated with 100 μl of monoclonal antibody for 1 h on ice, washed for 5 min each with PBS, 0·5 M-NaCl, and PBS, then all cultures were incubated with 100 μl of affinity-purified peroxidase-conjugated goat anti-mouse Ig (Kirkegaard-Perry Labs) diluted 1:7 in NKH buffer (144 mM-NaCl, 5·3 mM-KCl, 15 mM-HEPES/NaOH pH 7·3) with 1 % gelatin for 45 min on ice and washed as above. Development of the stain with diaminobenzidine is as previously described (Bechtol, 1984).

Treatment of cultures with antibodies

Monoclonal antibodies were used as they were harvested from serum-free culture of the hybridoma, depleted of transferrin by passage over an anti-transferrin affinity column (Bechtol *et al.* 1985) or purified from serum-free supernatant on protein A (Bechtol *et al.* 1985). In all cases, the antibody solution was dialysed overnight against SF/F12/DMEM plus supplement, filter-sterilized (Millipore, 0·2 μm) and diluted into SF/F12/DMEM plus supplement. Sister cultures in 24-well plates were treated in duplicate with antibodies, and the morphological appearance of cultures was observed at ×400 and recorded in Tri-X film (Kodak) with a Wild M-40 inverted microscope. Alternatively, the treated cultures were washed with PBS, fixed for 30 min at 4°C with 1 % glutaraldehyde in PIPES buffer, rinsed with PIPES buffer, embedded in EPON 812 and sectioned for electron microscopy (EM). Sections were observed in a Zeiss M-10

EM and recorded with Kodak EM film. Results were identical with all three types of antibody preparation.

Preparation of Fab fragments

Fab fragments of XT-I-antibody and HB-11 were prepared by incubating protein A-purified antibodies with 2% (w/w) papain (Worthington) in 0·01 M-cysteine and 2 mM-EDTA, pH 7·0, for 16 h at 37 °C. Papain digestion was terminated by dialysing the digest against 0·1 M-phosphate buffer, pH 7·2, for 1 h at 4 °C. Fab fragments were separated from Fc fragments and intact Ig by passage over a protein A-Sepharose column and collecting the unbound fraction. Alternatively, the fragments were run on an S200 column and the main ^{280}OD peak, minus the lower molecular weight shoulder, was used. When the Fab preparations were run under both reducing and non-reducing conditions on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and silver stained (Oakley, Kirsch & Morris, 1980; Bechtol *et al.* 1985), only monovalent Fab and light plus a fragment of heavy chain, respectively, were observed.

RESULTS

Immunoperoxidase staining with XT-I-antibody, identification of germ cells and their development in culture

In primary culture, Sertoli cells and germ cells migrate out of short tubule fragments to form a monolayer of flattened cells supporting clusters of round cells. To verify that the round cells in the culture system are germ cells, binding of the germ-cell-specific, developmental-stage-specific monoclonal antibody XT-I (Bechtol *et al.* 1979; Bechtol, 1984) was shown by indirect immunoperoxidase staining. Cultures from 10 days *p.p.* mice were terminated and stained 2 h after plating and at 24 h intervals. In the 2 h culture, no antigen-positive cells are seen (Fig. 1A). After 24 h in culture, a few scattered clusters of round cells begin to show cell surface staining indicative of increasing XT-1-antigen expression. Both positive and negative round cells can be seen in the upper outgrowth of Fig. 1B, consistent with the known presence of spermatogonia (XT-1-antigen-negative) and spermatocytes (XT-1-antigen-positive) in the same tubule segment. The clusters of round cells arising from the remaining tubule chunks are still negative (Fig. 1B). With each succeeding day in culture, the percentage of clusters staining with XT-I-antibody and the intensity of staining increase. The maximum number of positively staining clusters of cells is observed 4 to 5 days after plating (Fig. 1C), although occasional clusters of non-staining cells are still observable in these cultures. At this time antigen-positive cells represent approximately 50% of the total cell population. The three other monoclonal antibodies to the XT-1-antigen, MT-23, MT-24 and MT-29 (Bechtol *et al.* 1985), stain the developing cultures as does XT-I-antibody (data not shown). Binding of the antibodies in all cases is restricted to round cells and is not seen on the supporting epithelioid cells (Fig. 1A,B,C,E). Staining of cultures with HB-11, a monoclonal antibody of the same isotype as XT-I-antibody but binding H-2K^{b}D^b (Ozato & Sachs, 1981) labels the surface of the epithelioid cells faintly (Fig. 1D) but does not label the germ cells.

When 17 days *p.p.* mice are used to initiate cultures, the most mature germ cells present in the tubule segments are highly antigen-positive (Bechtol, 1984). This is

reflected in intense staining of germ cells in outgrowth fragments when tested at 24 h after plating (similar to Fig. 1C; data not shown). Thus, the detection of XT-I-antibody staining in the outgrowth cultures reflects the developmental age of the germ cells present.

Biological effect of antibodies

Primary cultures of germ cells were treated with the monoclonal antibodies. When 4-day cultures of testis cells from a 10 days *p.p.* mouse were incubated with XT-I-antibody, many of the germ cells formed tight masses with indistinct

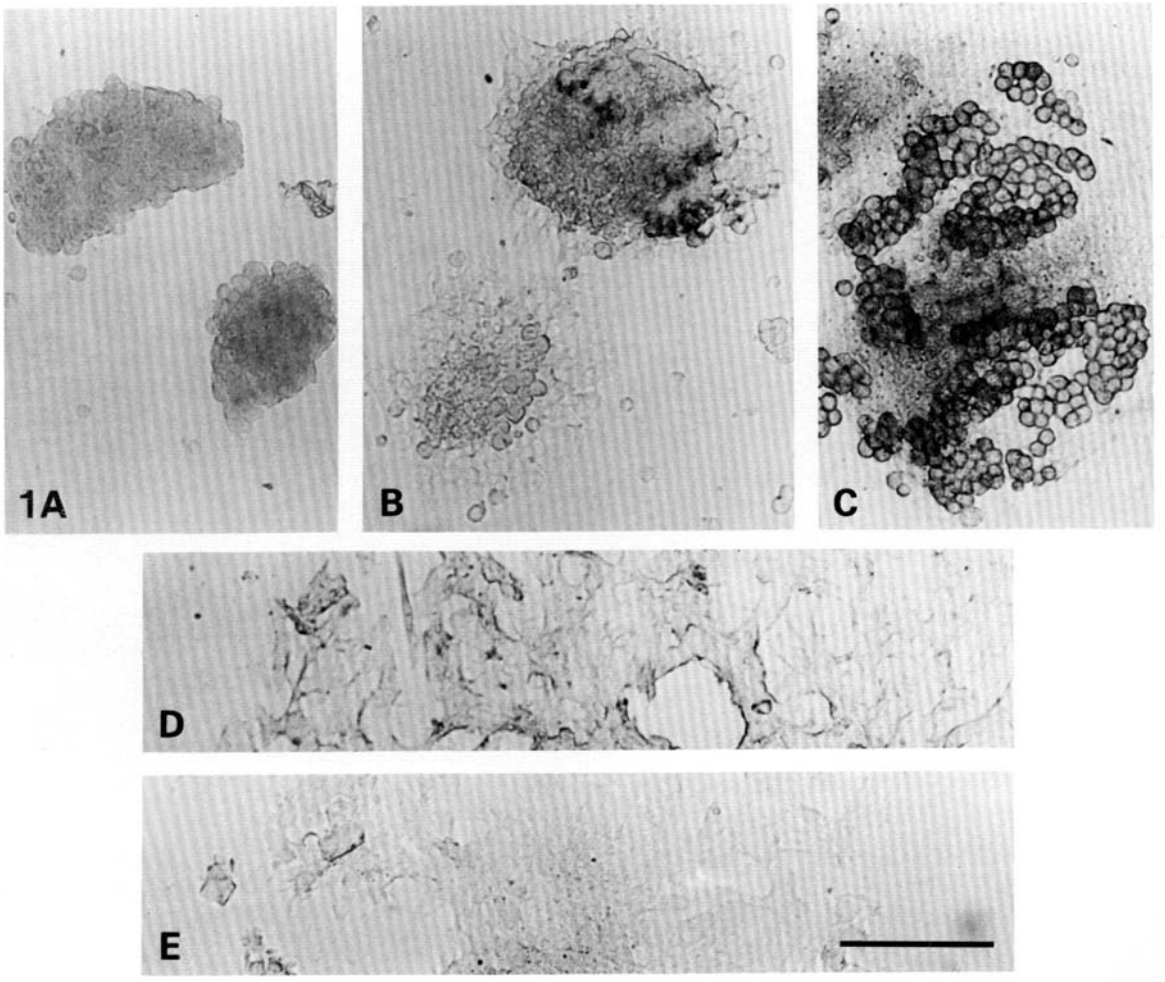


Fig. 1. Immunoperoxidase staining by XT-I-antibody (A,B,C,E) and HB-11 (D) in cultures of 10 days *p.p.* seminiferous tubules from strain 129 (A,B,C) and W^x/W^v (D,E) mice. Each stained cluster represents the most intensely stained cluster in that age culture. Cultures were fixed 2 h after plating (A), 24 h (B) or 96 h (C,D,E) after plating. Bar = 10 μm.

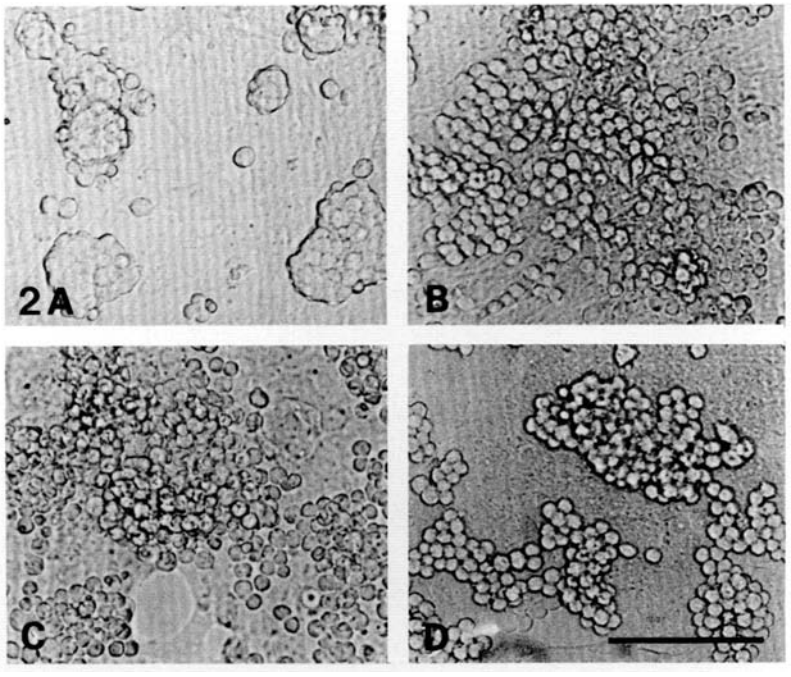


Fig. 2. Treatment of cultures with monoclonal antibodies. Tubule chunks from 10 days *p.p.* strain 129 mice were cultured for 96 h, then treated with XT-I-antibody (A), with MT-29 (B), with HB-11 (C) or left untreated (D). Bar = 10 μm.

individual cell boundaries. This is in contrast to cells in HB-11-treated or untreated wells which appeared as individual cells in grape-like clusters.

The response of cultures to XT-I-antibody is dose dependent. Treatment with 32 μg of protein A-affinity-purified XT-I-antibody per millilitre produces clumping of germ cells within 2 h (Fig. 2A) While lower doses require a longer period of time for the response to develop, as little as 0·044 μg of XT-I antibody per millilitre is effective after 24 h of treatment. If less than 0·004 μg of XT-I-antibody per millilitre is used, no detectable response is seen even after 96 h. In contrast, cultures treated with as much as 47 μg of affinity-purified HB-11 per millilitre (Fig. 2C) are indistinguishable from normal untreated cultures (Fig. 2D).

The XT-I, MT-23, MT-24 and MT-29 antibodies bind at least two distinct groups of epitopes (XT-I/MT-24, MT-23/MT-29) on the XT-1-antigen, as defined by the ability of each monoclonal antibody to inhibit binding of the other monoclonal to germ cells (Bechtol *et al.* 1985). Cultures were therefore treated with the MT antibodies to compare the binding map of the antigenic molecule to a 'biological map' of the antigen. When cultures were treated with equal binding activities (based on RIA) of MT-23 and MT-24, a result identical to that seen with XT-I-antibody (e.g., Fig. 2A) was observed. In contrast, cultures identically treated with MT-29 (Fig. 2B) over a wide range of antibody concentration were indistinguishable from untreated cultures. Moreover, prolonged treatment with MT-29 from the 4th day of culture to the 7th day of culture failed to produce a

detectable response. Thus, the biological map of the XT-1-molecule subdivides the epitope group MT-23/MT-29 defined by binding inhibition.

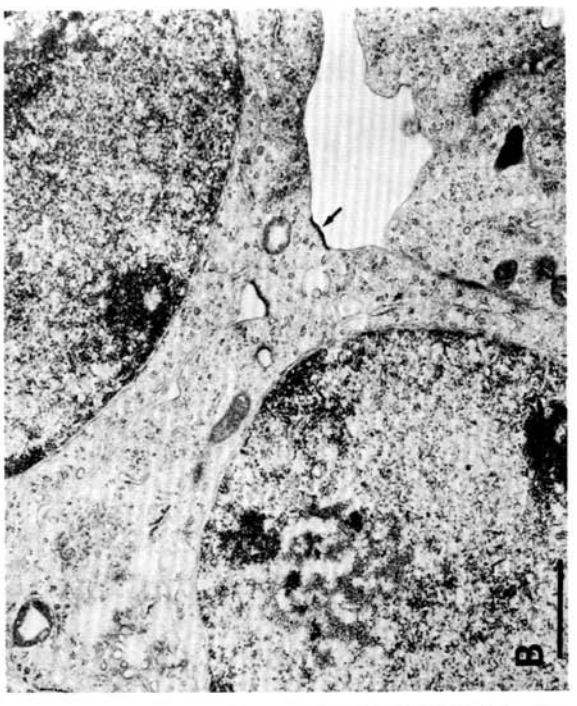
To test whether the effect of XT-I-antibody on germ cells is due to cross linking of antigen or cells by bivalent antibody, Fab fragments of XT-I-antibody were prepared by papain digestion. Cultures were treated with 133 $\mu g\,ml^{-1}$ to 0·18 $\mu g\,ml^{-1}$ of affinity-purified XT-I-antibody, 146 $\mu g\,ml^{-1}$ to 0·2 $\mu g\,ml^{-1}$ of the Fab fragment of XT-I-antibody, or 194 $\mu g\,ml^{-1}$ of affinity-purified HB-11 or HB-11 Fab fragment. Both whole Ig and the Fab fragment of XT-I-antibody produced clumping of germ cells within 2 h at high dose, while the effect produced by the lowest dose of each was observed at 32 h of treatment. As before, HB-11 failed to clump the germ cells. The response of germ cells to XT-I-antibody treatment is thus due to a mechanism other than cross linking of antigen or cells by the antibody.

Response in relation to germ cell age and XT-1-antigen expression

Cultures derived from 10 days *p.p.* mice do not respond to antibody treatment until the 4th day of culture, when they contain many highly antigen-positive cells. This is true whether antibody is added at the time of plating or on any of the succeeding days of culture up to the 4th day (data not shown). In the 4th-day cultures many of the groups of germ cells form tightly adhering clumps as described above (Fig. 2A). Germ cells in cultures derived from 17 days *p.p.* mice respond to antibody treatment as soon as the tubules are spread sufficiently to allow the assay (approximately 1 day, as in Fig. 2A; data not shown). Thus, the ability of germ cells to undergo antibody-elicited tight adhesion is a function of germ cell age and/or XT-1-antigen concentration.

Transmission EM of treated germ cells

Thin sections of germ cells treated with XT-I-antibody, with Fab of XT-I-antibody or with HB-11 were observed using transmission EM. Normal, intact cytoplasmic bridges were seen frequently in all cultures (Fig. 3A,B), with no evidence of multinucleate cells. This rules out the possibility of opening of cytoplasmic bridges. But EM did reveal a striking difference between cultures responding to XT-I-antibody (whole Ig or Fab) and cultures treated with HB-11 or left untreated. In cultures treated with HB-11, apposition of neighbouring germ cells is slight and is consistent with the normal packing of round cells (Fig. 3A,C). In XT-I-antibody-treated cultures, however, there is a large increase in close contact between membranes of adjacent germ cells. This results in a distortion of the germ cells to allow for the increase in cell–cell contact (Fig. 3B,D). In some areas finger-like extensions are present, indicating that the close contact between cells is a very favourable arrangement. Furthermore, it is frequently observed that germ cells along the edge of a cluster have formed extensions over adjacent cells (Fig. 3D). This phenomenon translates directly into the clumped appearance of treated cells as seen under the light microscope.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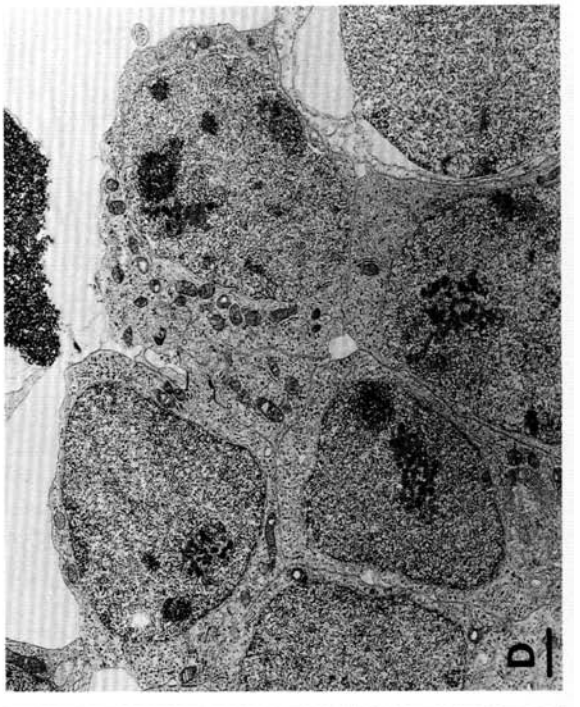
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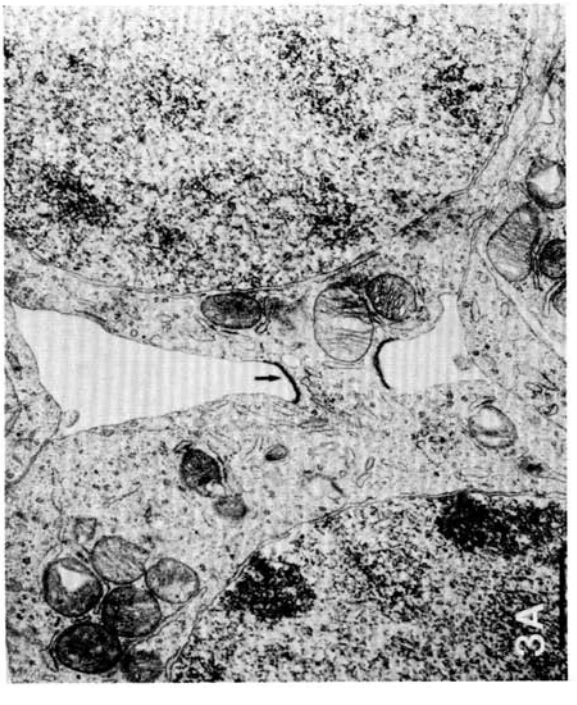
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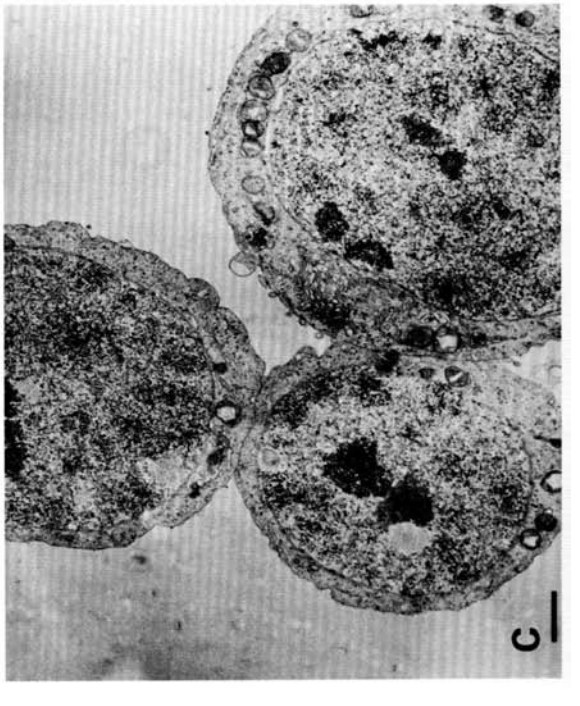
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DISCUSSION

The control of cell–cell adhesion plays a crucial role in many developmental systems, for example in embryo compaction (Damsky *et al.* 1983). However, little is currently known of the mechanisms by which adhesion is regulated. The XT-1-antigen system provides a novel approach to this problem; it is the first reported case in which antibody increases adhesion, other than the trivial case of multivalent antibodies acting as the cross-linking agent between cells.

Control of cell–cell adhesion is important in the *in vivo* germ cell environment. In the testis tubule, germ cells are almost completely surrounded by thin extensions of Sertoli cells that effectively separate each germ cell from its neighbours, except at the cytoplasmic bridges between sister germ cells (Fawcett, 1975; Wong & Russell, 1983). This argues that germ cell–germ cell adhesion is not favoured *in vivo* relative to germ cell–Sertoli cell adhesion. Also, under the physical pressure within the tubule, Sertoli cells may flow to fill all available spaces. Germ cells receive all nutrients by transport through Sertoli cells; thus a mechanism to ensure that germ cells do not exclude Sertoli cell extensions by adhering to each other would be highly advantageous. In cultures of tubule outgrowths, germ cells do not adhere to each other extensively but remain as distinct round cells, unless antibody is added. Transmission EM of germ cell clumps produced by XT-I-antibody (whole Ig or Fab) treatment revealed normal, intact cytoplasmic bridges connecting germ cells and increased germ cell–germ cell adhesion. The most straightforward conclusion is that XT-1-antigen is involved in a mechanism that normally blocks germ cell–germ cell adhesion.

By Scatchard analysis the mean number of XT-I-antibody binding sites per late pachytene spermatocyte is of the order of 2×10^6 (Bechtol *et al.* 1985). Using this as an approximation of the antigen present, assuming 50 % antigen-positive cells in the 4-day cultures, and using the K_0 calculated for binding at 0 °C, the expected per cent saturation of available antigenic sites at each antibody dilution can be calculated. By this estimate, production of a clumping response detectable in the light microscope at 24 h requires saturating or near saturating amounts of antibody. This is in contrast to the effects of specific antibody to some cell surface receptors [viz. insulin receptor (Kahn, 1979); mast cell IgE (Raff, 1975; Lawson, Fewtrell, Gomperts & Raff, 1975)], where only small amounts of bivalent antibody (less than 10 molecules per cell) are required to cross link a few receptors and induce a response. In those two systems, monovalent antibody fragments, even in high concentration, failed to produce a biological effect. The results described here using monoclonals that bind the XT-1-antigen are consistent with perturbation of a function that is performed by summation of the activity of many of the XT-1-molecules present on the cell. It may be that young germ cells, with fewer XT-1-molecules on their surface, do produce small areas of tight adhesion,

Fig. 3. Transmission electron micrographs of germ cells cultured for 96 h, then treated for 24 h with HB-11 (A,C) or XT-I-antibody (B,D). Note the increased cell membrane apposition in the XT-I-antibody-treated cultures compared to the HB-11-treated cultures. Arrow points to cytoplasmic bridge between germ cells (A,B). Bar = 1 μm.

but are incapable of producing large areas of adhesion sufficient to cause light-microscopically visible distortions in cell shape. The formation of tight adhesion is a consequence of antibody binding and is not the result of cross linking since a monovalent Fab fragment also induces the clumping response. Furthermore, treatment with the antibodies that recognize the XT-I/MT-24 and MT-23 epitopes, but not the MT-29 epitope, on the XT-1-molecule results in increased germ cell adhesion. Such epitope-specific effects have been found using groups of monoclonal antibodies that recognize distinct epitopes of the acetylcholine receptor (Richman, 1984) and also with a set of monoclonals that bind the lymphocyte function associated-1 (LFA-1) antigen (Ware *et al.* 1983).

The XT-1-antigen could function and be affected by a specific antibody through one of several mechanisms. For example, the XT-1-molecule may be an adhesion molecule similar to the cell surface lectin on epididymal sperm described by Shur & Hall (1982*a*). The ligand specificity of this lectin, and thus the adhesion specificity of the cell, can be modified by binding α-lactalbumin to the lectin (Shur & Hall, 1982*b*). Using this lectin ligand interaction as a model we can postulate that the three monoclonal antibodies that elicit germ cell tight adhesion may do so by stabilizing, or destabilizing, the association of an α-lactalbumin-like molecule with the lectin/antigen or by mimicking the effect of such a molecule on the lectin/antigen. Alternatively, the XT-1-antigen may be a protease or glycosidase which can enzymatically alter either an adhesion molecule or its target. If the monoclonal antibodies mask or activate (Frackelton & Rotman, 1980) such an enzymatic activity of the XT-1-antigen, they could create a functional adhesion molecule or modify the structure of a potential adhesion-mediating ligand. Yet another possibility is that XT-1-antigen does not participate directly in adhesion, but may induce the adhesion mechanism indirectly, through perturbation of the cell membrane or cytoskeleton. Such non-specific rearrangement of the cell membrane could expose previously buried adhesion sites. It might be that the Fab-elicited adhesion system we have observed normally functions during a discrete period in germ cell development, for example, in retention of the residual body during release of mature spermatids. Ultimately, understanding the mechanism of Fab-elicited adhesion and its normal *in vivo* control will enhance our understanding of the ways in which cells regulate their adhesion during development.

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