

A practicable immunological approach to block spermatogenesis without loss of androgens

(bacillus Calmette-Guérin/plasma androgens/libido/control of male fertility/azospermia)

G. P. TALWAR, R. K. NAZ, C. DAS, AND R. P. DAS*

Department of Biochemistry, All India Institute of Medical Sciences, New Delhi-110016, India; and *Department of Reproductive Biomedicine, National Institute of Health & Family Welfare, New Delhi-110016, India

Communicated by Vultimiri Ramalingaswami, July 17, 1979

ABSTRACT The intrinsic capacity of the immune system to elicit immune response selectively against late developing sperm proteins has been mobilized to intercept spermatogenesis. *Bacillus Calmette-Guérin* given in appropriate doses intratesticularly is effective in bringing about this effect. In dogs and rhesus monkeys, the sperm count in the semen declined precipitously, and almost complete azospermia was attained in 4-6 weeks after immunization. The few sperm cells that were present were immotile. Examination of serial sections of testes in immunized rats showed about 98% of the tubules to be devoid of sperm. The tubules were partially or fully atrophied. The basement membrane was, however, intact and the peritubular cell layer was normal. Sertoli cell nuclei were apparently normal but the cytoplasm was vacuolated and, in most cells, partially disintegrated. The lumen of the tubules was exhausted of formed elements and at times filled with eosinophilic debris. Leydig cells were present and hyperplasia of interstitial cells was seen, with massive infiltration of leukocytes. Blood testosterone levels were in the normal range and Leydig cells were responsive to gonadotropins. Libido was intact. The method was applicable to a variety of mammalian species. The implications of the results are discussed.

Numerous attempts are being made to develop new methods for control of male fertility. Progestogens have been used to suppress the production and, possibly, the maturation of sperm cells (1-4). Dosages required to achieve azospermia, however, appear to vary widely from individual to individual; pregnancies have been recorded in couples in whom the male partner was seemingly on high dose, which, however, produced in him only oligospermia and not azospermia (4). Intake of these steroids is accompanied by undesirable side effects, such as loss of libido (4, 5), presumably by virtue of their antiandrogenic action (6), thus demanding the supplementation of the regime with androgens (7).

As an alternative to steroidal contraception, the feasibility of immunization against a number of sperm antigens is being explored. The most promising of the sperm-specific antigens are LDH-x (8) and acrosin (9). A major limitation in their practical use is the difficulty in producing potent immune response in iso- or heterospecies; in spite of recourse to strong adjuvants such as Freund's complete adjuvant, a reduction, but not complete block, of fertility is obtained in test animals (10).

This communication describes a simple and effective procedure for achieving aspermatogenesis in mammals. The method is based on the rationale that the body's immune system is tolerant to only those testicular constituents that are made and functional during fetal life. New proteins emerging with the onset of spermatogenesis in postnatal years are "foreign" to the immune system and intrinsically elicit immune response if the opportunity is given to do so. The validity of this hypothesis is

supported by the frequent formation of antisperm antibodies in subjects undergoing vasectomy operations (11), a situation in which sperm cells escape the testicular barrier and are within reach of the immunocompetent cells. Indeed, autoallergic orchitis has been produced in the past (12-16) by injection of testicular extracts and Freund's complete adjuvant. This adjuvant, however, is not a permissible one for eventual male use. We propose the use of a possibly acceptable agent that gives the immunocompetent cells access to tubules and renders the tubules largely free of spermatozoa. In arriving at this agent, several other considerations were kept in mind. (i) It should be effective in most, if not all, of the recipients; (ii) It should be an agent that has been approved for human use and about which clinical experience over several decades is available. This will shorten the estimated 10-15 years considered necessary by experts for safety studies on a new contraceptive. (iii) The same agent should be effective not only in primates, but also in common laboratory animals so as to make possible large-scale safety studies at minimal cost. The requirements for baboons, apes, and homologous β subunit of choriogonadotropin have been a serious limitation to the speedy development of vaccines against human choriogonadotropin (17, 18) and have added enormously to the cost. (iv) Last, but not the least important, would be the desirability of imparting additional immunoprophylactic benefits of therapy to the recipient besides the control of fertility.

MATERIALS AND METHODS

Reagents. *Bacillus Calmette-Guérin* (BCG) was either from BCG Vaccine Laboratories (Madras, India) or a product of the Connaught Laboratories (Toronto, Ontario). The lyophilized material was reconstituted in sterile, pyrogen-free isotonic saline (0.9% NaCl, wt/vol). A unit of BCG contained 10^8 bacilli (from Madras) or consisted of 0.1 mg of lyophilized material (from Connaught Laboratories).

Immunization. Rats were given a single injection of 7.5 units of BCG in 0.2 ml of saline in each testis with a 25-gauge needle. In dogs, 110 units of BCG suspended in 1.5 ml of saline was given in a single injection into each testis. In rhesus monkeys, 100 units in 1.5 ml was given bilaterally. The needle was inserted from one pole of the testis and pushed gently to the other, with the material deposited at various points. The controls were injected with an equal volume of isotonic saline.

Semen Analysis. Semen was obtained from dogs by masturbation. The semen from rhesus monkeys was collected by the electroejaculation technique of Mastroianni and Manson (19) by use of two penile electrodes. The semen was incubated at 37°C for 30 min for liquefaction.

Sperm Counts. A Neubauer counting chamber was used for counting sperm at various dilutions of the semen (1:2, 1:10, 1:50, or 1:100).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: BCG, bacillus Calmette-Guérin.

Spermatozoan Motility. A drop of fresh or liquefied semen was taken on a slide and covered with a cover slip; five fields were counted under high power. The motility was expressed on a 0–5 scale, where 0 indicated no motile sperm, 1 indicated 20% of sperm motile, 2 indicated 40% of sperm motile, and so on.

Spermatozoan Viability. One drop of the semen after liquefaction was incubated at 37°C with a drop of eosin/nigrosin stain. A thin smear was prepared on a clear slide, air dried, and mounted in DPX mountant (BDH chemical). One hundred cells were counted under oil immersion. The dead sperm cells stained with eosin; living cells did not take the dye.

Histology. The testes, after dissection, were fixed in Bouin's solution. The tissues were transferred to a supersaturated solution of lithium carbonate in 70% alcohol for 24 hr, followed

by passage through graded dilutions of alcohol. Paraffin blocks were made, and 5- to 6- μ m-thick deparaffinized sections were stained with either hematoxylin/eosin or periodic acid Schiff's stain/hematoxylin.

Testosterone Estimation. Rats were bled by retro-orbital puncture with glass capillaries. Blood was drawn in heparinized syringes from veins of fore or hind limbs in dogs and monkeys. Plasma testosterone was estimated by radioimmunoassay.

RESULTS

The method was found to be applicable to a variety of mammalian species. It has been tested successfully in 98 rats, 24 guinea pigs, 13 rabbits, 18 dogs, 3 rams, and 3 rhesus monkeys. Typically, a single injection of BCG, in adequate doses, caused the tubules to be largely free of sperm cells. An equal volume

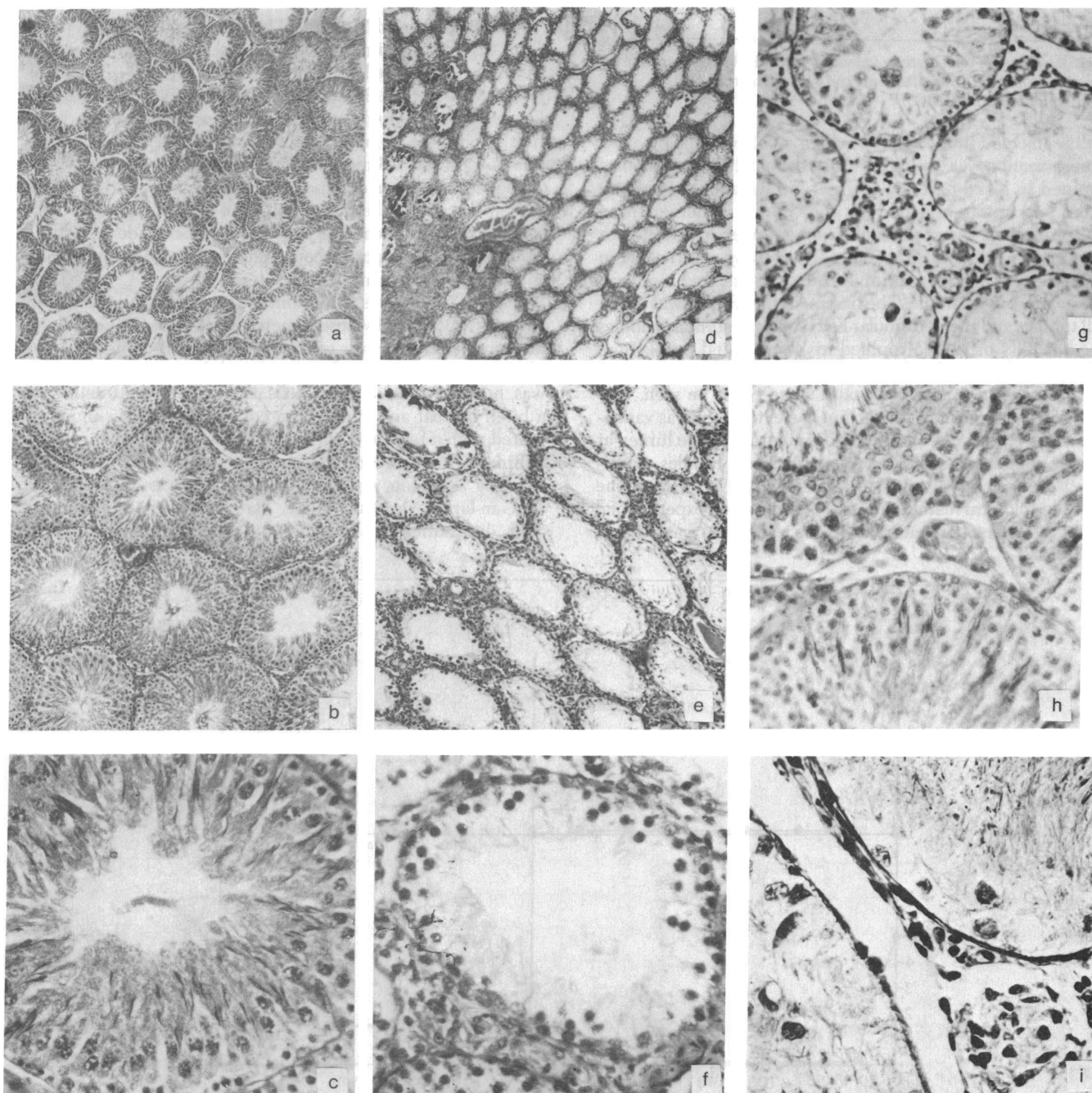


FIG. 1. Photomicrographs of rat testes immunized with 7.5 units of BCG on day 76 (*d–f*) and day 28 (*g* and *i*) after immunization. Sections of testes from normal rats injected with saline only and processed under identical conditions are included for comparison (*a–c* and *h*). *a–f* and *h* are stained with hematoxylin/eosin; *g* and *i* with periodic acid/Schiff stain/hematoxylin. (*a* and *d*, $\times 100$; *b* and *e*, $\times 250$; *c*, *f*, and *h*, $\times 1000$; *g*, $\times 500$; and *i*, $\times 1250$.)

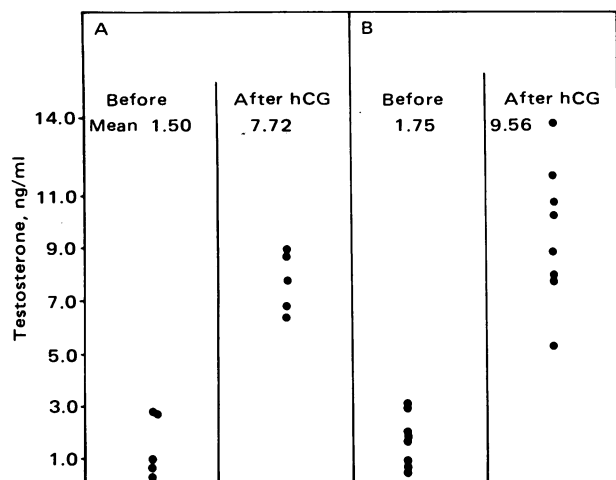


FIG. 2. Plasma testosterone values in control (A) and immunized (B) rats before and 6 hr after intramuscular injection of 7.5 μ g of human gonadotropin (hCG).

of saline injected in the same way had no effect. The block to sperm formation was complete in approximately 98% of tubules examined in serial paraffin sections in rats; about 2% of the tubules had apparently normal appearance. Whether these were producing sperm in significant number is difficult to discern from histological sections. The few sperm that were found in the semen of immunized dogs, rams, and monkeys were invariably immotile. The basement membrane was intact in all tubules and the peritubular cell layer was normal. Tubules were partially or fully atrophied in almost equal proportion. In the partially atrophied tubules, different types of germinal cells up to, at most, the spermatid stage could be seen. The Sertoli cells had normal nuclei, but the cytoplasm was vacuolated and, in most cells, partially disintegrated. The lumen at times contained eosinophilic debris. In a few tubules, multinucleated giant cells were seen occasionally. Fig. 1 shows the typical histological features of testes in control and experimental rats. In the interstitial spaces, leukocyte infiltration was evident.

The Leydig cells with normal cytoarchitecture were discernible (Fig. 1i). Whether their number was increased is difficult to ascertain with certainty. In most cases, hyperplasia of interstitial cells was seen.

Leydig cell function was not impaired, as gauged by random plasma testosterone levels, which were in the same range as in control animals (Fig. 2). To exclude the possible contribution to blood of androgens from the adrenal cortex (instead of from the testes), we determined blood testosterone in immunized rats after injection of a load dose of gonadotropin. This hormone selectively stimulates steroidogenesis in the Leydig cells but does not stimulate steroidogenesis in the adrenal cortex. A rise in blood androgen level was seen in all immunized animals (Fig. 2). Similar results were obtained in several other species of animals, complete data on which will be described elsewhere.

Sperm Count and Motility. The sperm count in semen declined rapidly after immunization. In general, very low sperm counts were obtained in semen samples after 4–6 weeks of immunization. Figs. 3 and 4 are representative examples in a dog and a rhesus monkey. The few sperm, when present, were mostly immotile. Semen remained free of sperm in these animals for almost 6–8 months. Long-term suppression of spermatogenesis was thus obtained in these species. It is difficult to state at this stage whether the block of spermatogenesis is reversible or permanent. Perhaps it could be made reversible either by intrinsic spontaneous regeneration and repair or by chemotherapy with antimycobacterial drugs.

Libido. Dogs, rams, and monkeys immunized intratesticularly with BCG and whose semen lacked or contained a very low number of immotile sperm cells did not demonstrate lack of libido. Rams mounted on ewes and donated semen which was, however, free of sperm cells. Dogs retained attraction for a bitch in heat and engaged in copulation. Rhesus monkeys mated with a female in the estrus phase of the cycle.

Side Effects. A side effect of the procedure was scrotal swelling and pain, especially in animals given high doses of BCG in large volumes of saline. These effects were minimized with diminution of the injected dose. Local effects usually

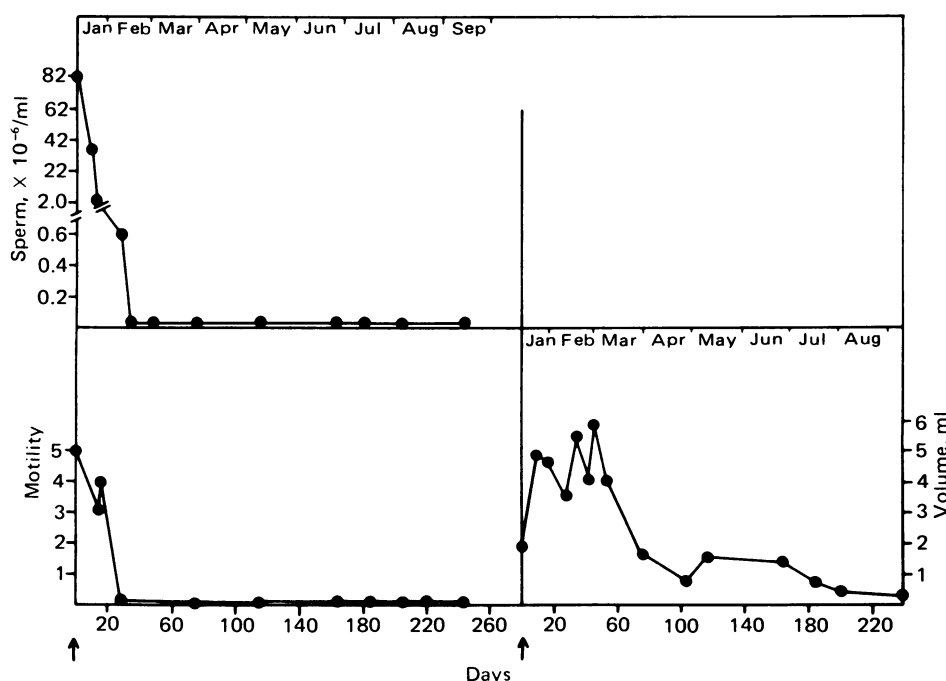


FIG. 3. Analysis of semen of a dog on different days after immunization with 110 units of BCG. The dog mounted and copulated normally with a bitch in heat on the 34th day after immunization. The copulation was, however, infertile. Arrows, day of injection of BCG.

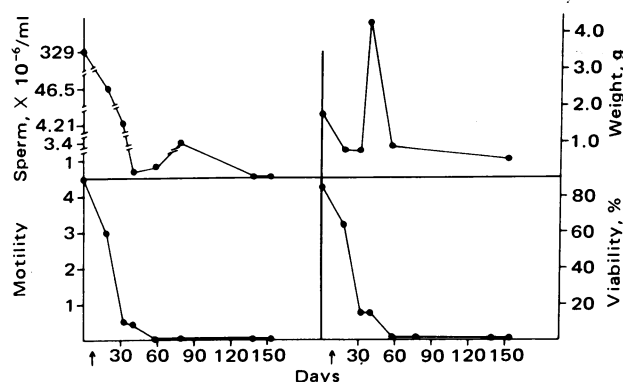


FIG. 4. Effect of intratesticular immunization with 100 units of BCG on sperm count, motility, viability, and weight of the semen from a rhesus monkey. Arrows, day of injection.

subsided within 2 weeks in dogs. The local swelling and pain as a result of immunization were not noted in the three rhesus monkeys investigated, who were given 50, 100, and 160 units per testis in 1.5 ml of saline.

The present mode of immunization is otherwise largely free of systemic effects. A transient pyrexia was observed in rams at very high dose, but was not noted in dogs and other animals on low and moderate doses. The weight record of the immunized animals was indicative of lack of morbidity. Assays of blood did not reveal significant alterations in serum proteins, glucose, cholesterol, urea, creatinins, and bilirubin. Hematological values like hemoglobin and total and differential leukocyte counts remained in the normal range. Detailed data on various species of animals will be reported elsewhere.

DISCUSSION

Satisfactory control of male fertility is considered to be achieved only in situations in which viable motile sperm are fully prevented from insemination in the female genital tract. A method that produces only partial decrease in production (oligospermia) or partial killing, as is the case with many spermicidal preparations, fails to impart fool-proof protection. One of the merits of the method proposed in this communication is its ability to produce nearly complete azospermia. The few sperm cells present in the ejaculate were immotile.

Intervention at the site of production, as achieved in this method, may be more reliable than immunological interception through antibodies or sensitized cells in the female genital tract. Leaving aside the difficulty in producing high enough immune response in the female animal against sperm antigens without recourse to strong and impermissible adjuvants, there will be the uncertainty of inactivation of 100% of the spermatozoa by the antibodies during the transit of the spermatozoa in the genital tract. A single ejaculate may contain 30 million to 300 million sperm, and just a single sperm, reaching the vicinity of the ovum in good state, can potentially cause fertilization. Iso-immunization against sperm antigens is thus likely to have practical difficulties of logistics in spite of laudable merits. This is avoided in the present approach of eliciting an autoimmune reaction against proteins developing with spermatozoa.

The dosages of BCG required for inducing damage confined to germinal cell development should be worked out for each species. Massive doses can bring about nonspecific tissue damage, obliterating the basic structure. Previous sensitization to BCG may reduce the dose requirement. The response, when regulated properly with appropriate dose of the material, is exercised selectively against only those constituents to which

the immune system is not tolerant and which are not present (or operative) during the fetal life. Thus, the basement membrane, Leydig cells, and other structures made in embryonic life are spared. By the same logic, it is expected that the immune response generated will be discriminatory and will not operate against other tissues considered as "self" components.

The choice of BCG for provoking autoimmune aspermatogenesis was arrived at after experimenting on almost a dozen other agents. The bacilli were effective in the killed state, but the dose requirement was much higher and the action was localized around the point of deposition.

There may be additional benefits of using BCG. Guinea pigs rendered aspermatic by intratesticular BCG developed delayed hypersensitivity to tuberculin. Besides protection against tuberculosis, BCG is an adjuvant and has the property of stimulating, in a general manner, the reticuloendothelial system, with resultant improvement of immunological capacities against infections. BCG therapy has also been reported to be beneficial in cancers (20).

The present method spares surgery. Its side effects, if any, need to be carefully studied in model experimental animals. An advantage is the lack of fall in blood testosterone levels and retention of libido in a situation where spermatogenesis is nearly completely arrested.

We thank Jatinder Sandhu, H. K. Sharma, and M. G. Sharma for help. This work was supported by grants from the Family Planning Foundation of India and from International Development Research Centre of Canada through the International Committee for Contraception Research of the Population Council Inc., New York.

1. Prasad, M. R. N., Singh, S. P. & Rajalakshmi, M. (1970) *Contraception* 2, 165-178.
2. Roy, S., Chatterjee, S., Prasad, M. R. N., Poddar, A. K. & Pandey, D. C. (1976) *Contraception* 14, 403-420.
3. Frick, J. (1973) *Contraception* 8, 191-206.
4. Schearer, S. B. (1978) *Int. J. Androl.*, Suppl., 2 (abstr.).
5. Saksena, S. K., Purvis, K., Cekan, Z., Diczfalussy, E., Giner, J. & Paulsen, A. C. (1975) *Acta Endocrinol. (Copenhagen)* Suppl. 199, 294.
6. Johansson, E. D. B. & Nygren, K. G. (1973) *Contraception* 8, 219-226.
7. Brenner, P. F., Mishell, D. R., Jr., Bernstein, G. S. & Ortiz, A. (1977) *Contraception* 15, 679-691.
8. Goldberg, E. (1979) in *Recent Advances in Reproduction and Regulation of Fertility*, ed. Talwar, G. P. (Elsevier/North-Holland, Amsterdam), pp. 281-290.
9. Zaneveld, L. J. D., Schumacher, G. P. B. & Travis, J. (1973) *Fertil. Steril.* 24, 479-484.
10. Goldberg, E. (1974) in *Seventh Karolinska Symposium on Immunological Approaches to Fertility Control*, ed. Diczfalussy, E. (Karolinska Institutet, Stockholm), pp. 202-222.
11. Wartman, J. (1975) *Popul. Rep.* 2, 31-32.
12. Freund, J., Thompson, G. E. & Lipton, M. M. (1955) *J. Exp. Med.* 101, 591-603.
13. Freund, J., Lipton, M. M. & Thompson, G. E. (1953) *J. Exp. Med.* 97, 711-726.
14. Voisin, G. A. & Toullet, F. (1971) *Folia Allergol.* 18, 310-316.
15. Mancini, R. E., Andrada, J. A., Saraceni, D., Bachmann, A. E., Lavieri, J. C. & Nemirovsky, M. (1965) *J. Clin. Endocrinol. Metab.* 25, 859-875.
16. Katsch, S. (1964) *Int. Arch. Allergy* 24, 319-331.
17. Talwar, G. P., Sharma, N. C., Dubey, S. K., Salahuddin, M., Das, C., Ramakrishnan, S., Kumar, S. & Hingorani, V. (1976) *Proc. Natl. Acad. Sci. USA* 73, 218-222.
18. Talwar, G. P. (1979) *J. Reprod. Biomed.* 22, 61-76.
19. Mastroianni, L., Jr. & Manson, W. A., Jr. (1963) *Proc. Soc. Exp. Biol. Med.* 112, 1025-1027.
20. Lamoureaux, G., ed. (1976) *BCG in Cancer Immunotherapy* (Grune and Stratton, New York).