Basic and biomedical product-oriented research—the National Institute of Immunology

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Five biomedical products have been licensed to industry in less than five years of work.

What started out some years ago around a working nucleus, an ICMR-WHO (Indian Council of Medical Research and World Health Organization) Immunology Centre in the Biochemistry Department of the All India Institute of Medical Sciences (AIIMS), and a barren patch of intractable land is today a full-fledged research institution with a firm commitment to research and development in frontier areas of the life sciences. With over 14 laboratories, 45 scientists, 45 Ph D students, and technical and administrative staff, the National Institute of Immunology (NII) in New Delhi has become a regional and international centre of excellence, with students and trainees coming from all over India and Iran, Afghanistan, Bangladesh, Sri Lanka, Nigeria, France and Brazil to work and to learn. The institute is designated as a Collaborating Centre of the WHO for Research and Training in Immunology for India and South-East Asia, a Centre for International Network for Molecular Cellular Biology of UNESCO and a Centre for Asian Network for Biotechnology Applied to Animal Production and Health of the UN's Food and Agriculture Organization (FAO).

Aims and objectives

NII was established to undertake, aid, promote, guide and coordinate research of a high calibre in basic and applied immunology. The main thrust of research is on problems of national relevance. Each one of them demands original, high-quality basic research. However, leads of potential utility are taken through product development and field testing, and converted to a stage of technology usable by industry for commercial production to benefit people. In less than five years of NII's functioning in its new campus, five products were licensed to industry.

Immunocontraception

Given the fact that the country's population is increasing at an alarming rate, now more than ever, there is a dire need for additional methods for family planning that are safe, effective, acceptable and suited to our socioeconomic

High-calibre research in basic and applied immunology

and cultural backgrounds. NII is engaged in developing novel methods for controlling human and animal fertility. Traditionally vaccines are made against pathogens bearing 'foreign' antigens; but the birth-control vaccines devised at NII aim to counteract a 'self' protein or a hormone critical to fertility. The strategy adopted to achieve this end will have a bearing on new immunological approaches to regulate other internal disorders such as insulin-dependent diabetes, thyroid hyperfunction and rheumatoid arthritis.

While the institute is working on a whole range of birth-control vaccines, an injectible that renders semen completely free of sperm (azoospermia) without causing loss of libido and decrease in levels of male sex hormone has already received New Drug Authorization from the Drugs Controller of India. It will be marketed under the trade name Talsur by the public-sector Karnataka Antibiotics and Pharmaceuticals Ltd in Bangalore. The injection is usable in all mammals but would be particularly useful for sterilization of bulls to stop the proliferation of animals of low economic value. Furthermore, by virtue of the fact that the bulls retain libido, they are usable as 'teaser' bulls to identify females in oestrus. Insemination with semen of high genetic stock at the right time enhances the chance of success of conception, as the fertile life of the egg is only one to two days. Thus Talsur would greatly help in animal husbandry and improvement of the genetic stock of animals. Talsur has also found application in sterilization of stray dogs.

Birth-control vaccine for women

Talwar (NII's director) *et al.* were among the first to propose the idea of a vaccine to control fertility. Its feasibility was demonstrated by the first prototype vaccine tried in non-human primates and in some women. The vaccine was so designed that it elicited antibodies against both tetanus and the pregnancy hormone human chorionic gonadotropin (hCG). Tetanus is a major killer of pregnant women in India in deliveries taking place outside hospital. The vaccine therefore has a double benefit. Immunization with this vaccine was found to be safe and free of any notable side effects. Women continue to ovulate and menstrual cyclicity is maintained. Its action is reversible, but can be prolonged with booster injections. The findings of phase-I clinical trials at five centres in India have been confirmed by similar trials in Finland, Sweden, Chile, Brazil and the Dominican Republic. The international trials were conducted under the auspices of The Population Council. An improved formulation of the vaccine has been cleared by drug regulatory authorities for phase-II clinical trials, which are in progress in three major centres in India. This is the first time anywhere in the world that a birth-control vaccine has reached this stage of development.

Synthesis and secretion of hCG by non-small-cell human lung cancer cells is another interesting observation reported in the literature. hCG serves as a growth factor for the tumour cells. Block of hCG synthesis by antisense RNA or its neutralization by antibodies results in inhibition of the growth of the cells into tumour mass *in vitro* and *in vivo* (in nude mice). There are no effective drugs against this type of cancer at present. The hCG recombinant vaccine of NII has been approved in two centres abroad for clinical trials in patients bearing hCG-making lung cancers.

Carriers such as tetanus toxoid (TT) and diphtheria toxin (DT) have served admirably to overcome the nonresponsiveness of women to hCG and gonadotropin releasing hormone (GnRH), which are self proteins. It has however been observed that repeated immunization using these carriers leads to immunosuppression. Experimental studies in genetically defined strains of mice demonstrate that this effect is subject to genetic restriction, a feature of immune response seen as variation in level of response among individuals depending on their genetic makeup in a well-defined region of the genome. A strategy has been worked out, employing alternative synthetic carriers, to circumvent the suppression. These studies point to the possible presence of epitopes within the TT and DT molecules that, instead of being 'helper', are 'suppressor' elements. These are being delineated with the objective of developing improved submolecular vaccines for tetanus and diphtheria, over and above the use of such peptides as carriers.

With the aim of making a low-cost vaccine deliverable to large numbers and requiring minimal repeat doses for generation of adequate response, NII is working on a recombinant hCG vaccine using vaccinia virus as a vector. Cells infected with the recombinant vaccinia virus, which carries the β-hCG gene linked to the membrane-anchor sequence of the vesicular stomatitis virus glycoprotein gene, express β-hCG on the surface. Monkeys immunized with this vaccine develop fairly high titres of anti-hCG antibodies and the antibody levels are sustained for several months. Vaccinated bonnet monkeys do not become pregnant during the period of high circulating antibodies.

There is also a great need for producing proteins of biomedical utility in large amounts by recombinant-DNA technology. The baculovirus expression-vector system, employing cultured insect cells as host or used *in vivo*, is an extremely powerful approach, particularly for expression of eukaryotic genes. At NII baculovirus recombinants have been engineered to express the genes for α- or β-hCG in insect cells. The recombinant hCG is bioactive and the level of expression is the highest reported so far for this hormone. Baculovirus vectors have also been engineered for simultaneous expression of more than one gene. NII is one of the four centres in the world, and the only one in India, with a demonstrated ability to use caterpillars as protein factories for high-level production of genetically engineered proteins in a cost-effective manner.

Mammalian cells are also being used as expression systems for genes of interest. An hCG–HBsAg (hepatitis B surface antigen) gene chimaera placed downstream of either the SV40 early promoter or the β-actin promoter expresses hCG in transfected LM tk$^-$ cells. CHO cells are also being used for the expression of genes such as hCG and ovine luteinizing hormone (LH).

Reproductive hormone-dependent cancers

Gonadotropin hormone releasing hormone (GnRH) controls production of the gonadotropins follicle-stimulating hormone (FSH) and LH, which in turn regulate production of the gametes (egg, sperm) and sex steroid hormones. As GnRH is the same in males and females, a vaccine inducing antibodies that inacti-

vate GnRH can regulate both male and female fertility. It can also be useful in cancers promoted by sex steroid hormones. Previous work from this institute has clearly defined the potential of a GnRH vaccine in blocking fertility. Indeed, clinical trials have begun in post-partum women, with the idea of extending lactational amenorrhoea and inter-child intervals. Another fascinating observation made was the drastic atrophy of the prostate following immunization of male rats and monkeys with the GnRH vaccine which indicated that this vaccine has therapeutic potential in hormone-dependent prostatic hypertrophy. After due toxicology studies and permission from the drug regulatory authorities and ethics committees, a GnRH vaccine is now undergoing phase-I phase-II clinical trials in patients of carcinoma of the prostate at AIIMS in New Delhi, the Post-Graduate Institute of Medical Education and Research (PGIMER) in Chandigarh, and in two centres abroad. Preliminary results suggest that this vaccine is indeed effective in reducing the size of the enlarged prostate and brings about improvement in clinical symptoms.

Basic research on the three-dimensional structure of molecules comprising the vaccines is expected to yield rich insights into regions recognized by the elicited antibodies and therefore lead to rational design of vaccines. NII's structural biology group is employing X-ray diffraction, computer graphics and molecular-imaging approaches to elucidate the structure of GnRH and hCG. Fab fragments of bioeffective monoclonal antibodies have also been crystallized.

Vaccines against sperm and egg

The spermatozoon is yet another interesting target in interception of fertility as it carries a number of autoantigens and isoantigens. From a human case of immunological infertility, a 40-kDa antigen located on the acrosome of sperm was identified Rats have a cross-reactive glycoprotein of 24 kDa. NII scientists have shown that immunization of rats and monkeys with a sperm-specific 24-kDa antigen results in the production of antibodies that prevent the attachment of sperm to the oocyte and a consequent reduction of fertility. Genetic-engineering methods are also being used to identify and isolate relevant genes of interest from testicular-cDNA libraries.

Another point of interception in the reproductive process is the zona pellucida. Experiments both here and elsewhere have shown that fertility can be blocked by immunization with zona pellucida antigens Purified zona antigens such as ZP3 are being used as candidate vaccines; monkeys immunized with highly purified ZP3 along with mild adjuvants fail to conceive for over eight cycles of observation, without any disturbance of cyclicity. To delineate the determinants on ZP3, monoclonal anti-ZP3 antibodies have been generated; these antibodies would also be of use in characterization of sperm receptors on the zona pellucida.

Plant extracts for contraception

Among India's potential contributions to the world is the wealth of medicinal herbs and plants. A recent NII finding on the ability of neem oil (from neem *Azadirachta indica*) to block fertility for several months after a single application in the uterus is of potential interest for developing a 'vacation' contraceptive. It will also be useful for covering the first three months of the latent period during which levels of anti-hCG antibodies are built up in the primary phase of immunization. Neem-oil treatment results in local cell-mediated immune reactions in the uterus. The treatment does not impair ovulation; hormone levels and libido remain undisturbed. In male rats, a single intraepididymal injection of neem oil results in abrogation of spermatogenesis, without testicular inflammation. Intracaudal injection of neem oil in monkeys leads to azoospermia without any discernible effect on serum levels of testosterone.

Embryo biotechnology

NII has an extensive and intensive programme of research and development in various areas of embryo biotechnology, exploiting current knowledge in embryology, endocrinology and molecular biology, for production of cattle of high genetic stock. The institute has to its credit three firsts in this area NII scientists were the first in India to produce a calf by non-surgical embryo transfer. This technology enables obtaining up to 150 cows from an 'elite' cow, in contrast to the about 10 that she would normally breed in traditional approaches. Scientists at NII also succeeded in producing a 100%-pure Holstein-Friesian calf using a local stray cow as surrogate mother The third achievement in this area is the perfection of embryo-splitting techniques and successful production of calves by transfer of 'split' embryos. Splitting has the advantage of doubling the yield of calves derived from each embryo. New methods for *in vitro* maturation, fertilization and development of buffalo oocytes to morula stage, when they can be transferred to recipients, have also been developed. The potential benefits of such advances to the cattle and dairy industry are obviously immense.

Work on molecular-biological aspects of embryo and animal biotechnology is focused on sexing of embryos using male-specific synthetic DNA probes, cloning and expression of LH, FSH, and ovine and bovine growth hormones (GH), and generation of transgenic animals. cDNAs for β-LH and β-FSH have been isolated from pituitary-cDNA libraries and characterized. Genes for GH and growth hormone releasing factors have been injected into mouse and rabbit eggs to obtain transgenic animals with better growth rate and feed-conversion ratio. Similar studies are in progress in fishes.

Another area of research concerns genetic and immunological analyses of Indian wild mice, which have been neglected by and large. This research is aimed at developing 'new' inbred strains of mice with potential for use as disease models in immunology and genetics.

Aquaculture has been a recent foray, with two major aims: induction of off-season breeding of freshwater fishes, and development of transgenic strains with better growth rate and feed-conversion ratio. Induced breeding of freshwater Indian major carps is being attempted with synthetic salmon-GnRH analogues, and preliminary results are encouraging The objective of these experiments is to increase production of nutritionally important fishes by inducing them to breed and proliferate even during periods when they do not normally breed

Communicable diseases

Research on communicable diseases is

focused on basic research and development of diagnostic methods and non-conventional or genetically engineered vaccines for bacterial, viral and parasitic diseases.

Leprosy continues to be a major scourge in India; epidemiological data reveal that there has been no significant decline in the incidence of leprosy in endemic areas, despite several decades of chemotherapeutic control programmes. Two crucial developments would help control leprosy—the availability of a reliable diagnostic test and a vaccine that can invigorate immunity in those who are deficient. A good vaccine may also contribute to therapy in multibacillary patients.

Some years ago, Talwar and his associates developed a vaccine based on an atypical cultivable non-pathogenic bacterium, *Mycobacterium w*, a member of the Runyon group IV family. *Mycobacterium w* (Mw) induces cell-mediated immune reactions, analogous to those induced by *M. leprae*, in tuberculoid-leprosy patients, and, more importantly, induces reactivity in lepromatous-leprosy patients who are normally anergic to *M. leprae*. Mw has strong immunogenic properties even when killed and induces *M. leprae*-cross-reactive delayed-type hypersensitivity (DTH) responses in animals. These features prompted exploration of the potential of Mw in the immunotherapy of leprosy. Phase-I clinical trials showed that immunization with killed Mw results in the conversion of lepromin-negative lepromatous-leprosy patients to a lepromin-positive status. This vaccine is well tolerated and induces a prolonged, stable DTH reactivity to *M. leprae*. Phase-II/III trials in clinically active cases of leprosy have yielded dramatic results—accelerated regression of lesions in vaccinated individuals as opposed to controls, rapid fall in the bacteriological index in vaccinated individuals, conversion of a majority of the immunized patients to lepromin-positive status, and marked improvement in the histopathology of immunized subjects. These encouraging observations have prompted large-scale phase-III/phase-IV clinical trials for assessment of the immunotherapeutic efficacy and immunoprophylactic potential of this vaccine in Kanpur Dehat covering a population of over 362,000 people.

A most interesting fallout of this study is the finding of the ability of Mw to confer protection in several genetic strains of mice against challenge with *M. tuberculosis* H37Rv, the virulent strain of the tuberculosis bacillus: Mw immunizes strains of both BCG-responder and non-responder mice against live *M. tuberculosis*. Protective efficacy is demonstrated by the fact that a significant reduction is observed in number and severity of tuberculoid lesions in the lungs following immunization with heat-killed Mw. This vaccine is also highly effective in guinea pigs, which are exquisitely susceptible to tuberculosis.

On the basic-research front, the mechanism of peripheral nerve damage in leprosy is being investigated. A primate model of leprosy has been developed in rhesus monkeys for the study of *M. leprae*-induced nerve damage. Lepromin-negative rhesus monkeys infected with armadillo-derived *M. leprae* developed dryness, scaling and ulceration, with resorption of terminal phalanges. Progressive nerve damage is observed as early as three months after infection and, interestingly enough, is accompanied, not by presence of acid-fast bacilli, but by the emergence of circulating anti-neural antibodies. With molecular immunology moving in the direction of elucidation of specific epitopes involved in inducing immunity, work on this front has led to the preliminary characterization of immunodominant epitopes of Mw.

For diagnosis of hepatitis B infections, a DNA-based probe for detection of hepatitis B virus (HBV) sequences in serologically negative sera is under development. Combining the powerful technique of the polymerase chain reaction with other molecular-biology methods, evidence has been obtained for the existence of variants of HBV that are not picked up by conventional serological methods. Some of these variants are being characterized. Point mutations and deletions of up to 200 bases have been noted.

A major form of hepatitis in India is caused by neither hepatitis A nor hepatitis B viruses, but by a so-called non-A, non-B hepatitis (NANBH) viral agent. An experimental monkey model for sporadic NANBH was developed from stool extracts from human cases of NANBH obtained from the Pasteur Institute in Paris, France; it was shown that the putative agent involved can be serially transmitted. The transmitted disease is characterized by elevated levels of serum transaminases and excretion of the agent in the stool. Stools of infected monkeys have infective particles, which, when separated by density-gradient centrifugation, can transmit the characteristic infection of sporadic NANBH to healthy monkeys.

A genetically engineered vaccinia-based rabies vaccine has been made by inserting the rabies virus glycoprotein G gene downstream of a vaccinia promoter. This live recombinant vaccine provides a high degree of protection against lethal intracerebral challenge with virulent rabies virus in all experimental mice tested so far. It is ready for further trials in dogs, which are the major reservoir of rabies and the principal source of infection to humans.

Immunodiagnostics

NII is developing several facile and rapid methods for immunodiagnosis of communicable diseases. An antibody-detection dipstick micro-ELISA has been developed for diagnosis of amoebic liver abscess. The assay takes about 20 minutes to perform and requires only a small amount of finger-prick blood. A similar test has also been developed for detection of *Salmonella typhi* infections.

A microplate ELISA for detection of anti-neural antibodies has been developed for accurate diagnosis of all categories of leprosy; it can discriminate leprosy from tuberculosis, dermatological diseases and autoimmune disorders. A recent advance is the development of a dipstick ELISA that can be performed in one hour.

Research is also under way for development of immunodiagnostic methods for HBsAg (sandwich enzyme immunoassay), *Streptococcus* A infections (latex agglutination assay), *Mycobacterium tuberculosis* (agglutination, immunofluorescence and immunoperoxidase assays) and *Entamoeba histolytica*. All these assays employ monoclonal antibodies generated at NII. The typing of red blood cells for ABO and Rh types is indispensable for blood transfusion. The production of absolutely reliable ABO- and Rh-specific polyclonal antisera is a time-consuming and tedious task, and monoclonal reagents are

currently being imported into India Monoclonal anti-A and anti-B blood group antibodies have been developed at NII with the objective of making available an indigenously devised kit for human blood grouping.

Nucleic-acid probes for diagnosis of tuberculosis and malaria are also being developed. For the diagnosis of tuberculosis, three different DNA fragments were isolated from a genomic library made from *Mycobacterium tuberculosis*. Two of these were found to be specific only to members of the tuberculosis complex and are being evaluated for their utility in detection of mycobacteria in sputum samples. Similarly, for the diagnosis of malaria, a test aimed at the detection of the over-abundant ribosomal RNA in the malarial parasite *Plasmodium falciparum* is under development.

Basic molecular biology

Research in basic molecular biology is directed at elucidating some fundamental biological mechanisms, besides the considerable attention focused on using genetic-engineering techniques for the development of vaccines and diagnostic methods described above. Experiments aimed at gaining understanding of the cellular machinery and the molecular signals involved in high-level expression, targeting and stability of heterologous proteins in baculovirus-infected insect cells and caterpillars use the firefly luciferase gene as a reporter gene. Experiments are also under way to identify *trans*-acting factors responsible for the temporally regulated and hypertranscriptional activity of the baculovirus polyhedrin-gene promoter.

Research aimed at analysing the regulation of mitochondrial-gene expression uses the unicellular eukaryotic organism *Chlamydomonas reinhardti*. Studies on the processing and editing of RNA in *Chlamydomonas* mitochondria have revealed remarkable conservation of these processes between simple eukaryotic organisms and complex vertebrates.

Considerable progress has also been made in development of novel DNA probes for DNA fingerprinting Four kinds of probes have been developed at NII A human satellite-DNA clone reveals polymorphism in the human genome with a number of restriction enzymes. A second kind is a synthetic oligonucleotide probe that reveals several highly polymorphic, individual-specific DNA bands. Another oligo probe was designed on the assumption that the genesis of hypervariability is recombinationally mediated, requiring, at the minimum, the presence of bacterial *chi*-like sequences. The usefulness of the *chi*-like homologues and of yet another, PCR-generated artificial mini-satellite probe in DNA fingerprinting is evident, and further experiments are focused on gaining understanding of the molecular mechanisms for generation of hypervariability.

End-to-end research

Basic and applied research at NII has already resulted in five products that have been licensed to industry. Two of the five have already been released to the public. For a product to reach this stage, basic research on a given concept is followed by generation of reagents such as monoclonal antibodies or genetically engineered molecules or chemically synthesized antigens. The method is put together and then evaluated in more than one laboratory at NII, followed by third-party development in other institutions and field agencies for evaluating efficacy, accuracy and specificity. Thereafter it undergoes 'product testing', in a specially created unit at NII, which aims at achieving stabilization of the reagents, introduction of quality-control procedures for reproducible manufacture, and development of functional packaging It is then offered to industry. The industry has its own schedule of making batches of reproducible quality based on technology transferred to it and market testing before the product is finally launched and made available to the public

Besides Talsur, NII's products are the following. A pregnancy-detection kit employing indigenously produced monoclonal antibodies has already reached the market through Ranbaxy Pharmaceuticals. Memoranda of understanding have been signed with a company for two other diagnostics: (i) a kit for detection of amoebic liver abscess, and (ii) a reliable test for the diagnosis of typhoid at the time the fever starts. The typhoid kit includes an easy-to-read colour indicator that advises treatment with the proper drug, to which the bacteria are sensitive. Anti-A and anti-B blood group monoclonal antibodies, described earlier, have also been licensed to industry.

Work on the vaccine front gives much scope for optimism. Progress on the hCG birth-control vaccine has been already described. The leprosy vaccine has shown immunotherapeutic potential over and above the expected immunoprophylactic benefit and has advanced from the hospital to a field area with a large population in a district with a leprosy prevalence rate of 25/1000. The GnRH vaccine is proving to be beneficial in patients of carcinoma of the prostate. Live recombinant vaccines have been made that engender high sustained antibody response against hCG and, in mice, protective immunity against rabies.

Recombinant cholera toxin B-chain has been expressed at high levels and is authentic in all respects. Molecular-genetic markers useful in sexing and DNA fingerprinting are on their way to commercial exploitation.

Funding sources

The National Institute of Immunology is an autonomous society deriving core funding from the Department of Biotechnology (DBT). Two-thirds of the working budget of NII is, however, met from competitive national and international research grants. Three major projects are part of the National Science and Technology Projects carried out in 'mission mode'. The birth-control vaccine research project also receives funding from the International Development Research Centre (IDRC) of Canada and The Rockefeller Foundation of the USA. Research on immunocontraception is also supported by grants from the US Agency for International Development (USAID) and the International Committee on Contraception Research of The Population Council, New York. Leprosy research projects have received grants from WHO and DBT. The institute has two projects funded from the Integrated Long-Term Programme of Cooperation in Science and Technology between India and the USSR. NII scientists are encouraged to apply for grants from national and international funding agencies. For example, three NII scientists are directly receiving grants from DBT and one is being

funded by the Indo-French Centre for Promotion of Advanced Research.

Though young in age, NII is firmly poised to fulfil its mandate of carrying out basic research of a high calibre and developing new vaccines, immunodiagnostics and other biomedical products of utility to the country.

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