An Experiment That Had to Succeed

Unexpected problems hindered the development of human monoclonal antibodies, but they were eventually overcome

Henry Kaplan and Lennart Olsson, of Stanford, were the first to report in the scientific press the production of human-human hybridomas that secreted monoclonal antibodies, although a team in Philadelphia produced similar cells at the same time. In an interview with *Science* Kaplan explained how serendipity helped him succeed.

In 3 months at the beginning of 1980, Lennart Olsson and Henry Kaplan cracked a problem with which biologists had been grappling unsuccessfully for 2 or 3 years. They developed a line of mutant tumor cells that would form the basis of production of pure, or monoclonal, human antibodies. "We really weren't aware of the enormous difficulties other people had experienced," Kaplan now says, "so when we embarked on the project we weren't particularly discouraged about the problems we might face."

This naiveté, together with an extraordinary set of propitious circumstances, led to the unusually rapid achievement of this much sought-after goal. "Once we had done it," he told *Science*, "a number of other research groups quickly succeeded in making similar cell lines. Our success persuaded them that it could indeed be done." In recent weeks, however, progress at Stanford has experienced a temporary setback through a problem that commonly afflicts cell culture work: contamination with the semiparasitic microorganism, mycoplasma. Luckily, prospects for overcoming the difficulty look good.

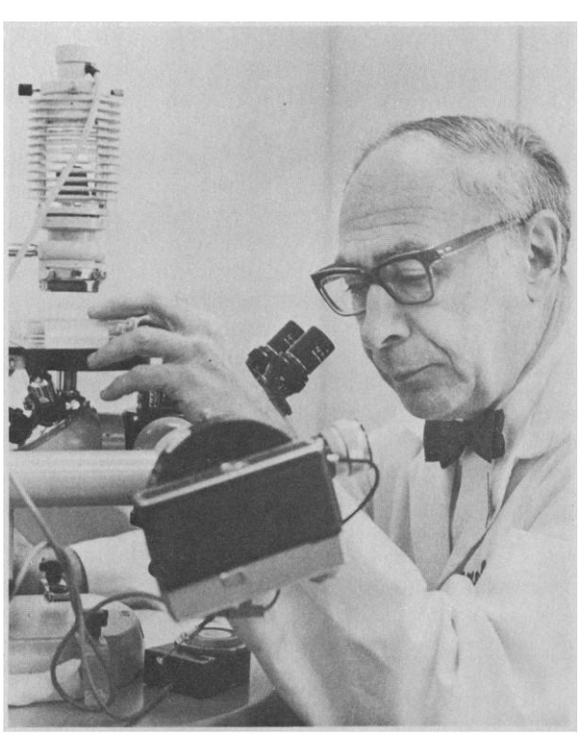
It is almost 6 years since César Milstein and Georges Köhler, of the Medical Research Council's Molecular Biology Laboratory in Cambridge, England, pioneered a method for manufacturing mouse monoclonal antibodies. This was an important event, because the potential of the technique in basic and commercial research is comparable with genetic engineering. An extension of the technique to include human antibodies was seen as inevitable, as it would offer new and extremely specific probes into many human diseases. The inevitable, however, took longer to arrive than anticipated.

Kaplan divides his time between basic research and clinical medicine at the Cancer Biology Research Laboratory and the Medical Center at Stanford University. Olsson, who has a background in immunology in Denmark and France, joined Kaplan for postdoctoral research around Thanksgiving, 1979. "My habit with new postdocs is to let them wander around the lab for a couple of weeks to see what's going on," says Kaplan. "Olsson did this, and then came to me with a shopping list of possible projects, one of which was to take a crack at making human hybridomas."

Hybridoma is the name given to the product of fusing together an antibody-producing cell (a B lymphocyte) and a cancerous form of a certain type of bone marrow cell (a myeloma). The hybrid has the virtues of both cell types: it manufactures an antibody with a single specificity, and it is virtually immortal. Milstein and Köhler had made the initial breakthrough by establishing these antibody factories using mouse cells. Human cells, it transpired, are not as tractable. The problem lay with obtaining a good line of myeloma cells with which the B lymphocytes could be fused.

As it happened, Kaplan, who as a physician is an authority in the treatment of Hodgkin's disease, had acquired a human myeloma line about 2 years before Olsson joined him. The cells, denoted U266, were of a type that several research groups, including Milstein's, had been trying to nurture as a possible component of the elusive human hybridoma. Kaplan, however, wanted them for quite different reasons, for experiments to do with viruses in certain types of cancer cells. "None of the experiments worked," recalls Kaplan, "so we just froze the cells in liquid nitrogen, and there they remained until Olsson took them out of cold storage 2 years later."

The preparation of the cells for those abortive experiments was crucial to the later success of the hybridoma work.



Henry Kaplan

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When they arrived from Kenneth Nilsson's laboratory in Sweden, the cells were not in the best of condition. For 4 months, Suzanne Gartner nursed the cells to peak health. "They were now healthy cells, growing faster than previously and with only a small percentage dying at each passage, and they could be cloned with a low but not negligible efficiency in agar or liquid culture," Kaplan recalls. Such was Olsson's inheritance when he pulled the cells from cold storage.

A crucial step in preparing myeloma cells for potential fusion is the production of a line of mutants that lacks the enzyme hypoxanthine phosphoribosyl transferase (HPRT). These cells die in a culture medium known as HAT. When the cells are mixed with lymphocytes for fusion, those that do not fuse are killed by subsequent exposure to HAT; the hybrids survive because the missing enzyme is provided by the lymphocytes. This is the system that Milstein and Köhler used in their work.

Olsson had no luck when he attempted to make the required mutants by exposing them to 8-azaguanine while spread out on agar plates. So he switched to the novel approach of using a liquid medium. He also decided to assault the cells with high levels of the chemical, quite contrary to normal practice. "He had a number of reasons for thinking this was the best approach," says Kaplan, "and it worked."

Next they needed to find a convenient source of antibody-producing cells. If you happen to be working with mice, the procedure is relatively straightforward. The mice are immunized with the desired antigen and, after a suitable period, the spleen is removed. The immunization provokes a strong proliferative response in those B cells that are programmed to respond to the specific antigen challenge. These proliferating B lymphocytes, which all essentially derive from a single parent cell and are therefore termed monoclonal, soon mature into plasma cells whose terminal function is to manufacture and secrete vast quantities of the one specific antibody. The spleen is therefore a rich source of the required antibody-producing cell.

Obtaining a source of human antibody-producing cells poses an obvious problem once again. But the Stanford team was well set to exploit good fortune. "I was in a unique position to have access to human spleens," explains Kaplan, "because of my involvement with Hodgkin's disease patients."

These people have a tumor of part of the immune system, and this may affect their immune defenses. Testing immune response of these patients by exposing them to 2,4-dinitrochlorobenzene has therefore been routine at the Stanford Medical Center for many years. Most normal people develop a skin reaction with this test, and they may also manufacture antibodies to a specific part of the molecule, the hapten dinitrophenol (DNP).

A second aspect of treatment of Hodgkin's disease is what is called staging laparotomy, which involves surgical exploration of the abdomen with the removal of the spleen. This has been carried out since 1961 at Stanford and, Kaplan says, "it has been extraordinarily helpful in our treatment program."

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Here then is the perfect natural "experiment." The patients are immunized with a specific known antigen: "This is something we do all the time with new Hodgkin's patients, so there's no ethical problem." And the patients' spleens are routinely removed after immunization. "So, we simply took the next three patients who came along and used the spleens as a source of primed antibody-producing cells."

Cell fusion is still something of an art rather than a precise science, but roughly speaking it involves the following idealized procedure. The spleen cells and mutant myeloma cells are mixed together with an agent that promotes cell fusion. (In fact, because of their state of imminent proliferation, the primed lymphocytes have a predilection to fuse.) The mixture is then exposed to HAT medium which kills all the nonfused myeloma cells. The nonfused lymphocytes soon die in culture as they do not possess the immortality of cancer cells. What remains is a collection of hybridomas, some of which produce the required antibody while others make different antibodies. When the solution containing these cells is distributed among many tiny incubation wells, there is a good chance that some of these minicultures will contain just one type of hybridoma. After a while, the liquid in which the cells are growing can be tested to see which antibody is being produced.

When Olsson and Kaplan embarked on this stage of their work they knew that they were entering poorly charted territory. "My conviction was that the whole process of making monoclonal antibodies was so complex, with so many places it could break down and fail, that it was absolutely essential for it to work the first time," recalls Kaplan. "Usually you don't feel that way about experiments. You say, we'll try it one way, and if it doesn't work, we'll try it another way. With this, there were so many ways it could fail, that if we failed we wouldn't know what to change. So I thought it was important to try to mimic the Köhler and Milstein technique as completely as possible."

The path trod by the Cambridge team was diligently followed, and after almost 2 weeks of anxious waiting to see if viable hybrids would be formed, and then careful testing to detect the expected type of antibody, the hoped for results were obtained. From the spleen of the first patient they isolated three clones of cells producing antibody to DNP, from the second patient two clones, and from the last none. "This told us that our system worked." This was in early March 1980. A paper was submitted to the *Proceedings of the National Academy of Sciences** at the end of June, and was published in September, the first such report to reach the scientific press.

(Although Kaplan and Olsson were first past the publishing post, another group, led by Carlo Croce at the Wistar Institute in Philadelphia also produced a human-human hybridoma early in 1980. They reported their work in the 4 December issue of *Nature*.†)

Since December more than 100 research groups throughout the world have received samples of the Stanford cells and 150 more are awaiting delivery. Time passed, and although Kaplan heard word of research teams making hybridomas, reports of monoclonal antibody production failed to appear. Within the past few months word began circulating that a number of people were experiencing problems with the cells. At about the same time Kaplan began to have difficulties with his own cells. "They weren't growing as well as they did initially and their fusion properties weren't as good," he reports. So, prompted by his own problems and word from other labora-

*L. Olsson and H. Kaplan, *Proc. Natl. Acad. Sci. U.S.A.* 77, 5629 (1980).

†C. Croce, A. Linnebach, W. Hall, Z. Steplewski, H. Koprowski, *Nature (London)* **288**, 488 (1980).

tories, Kaplan tested for the presence of mycoplasmas in late March. Positive.

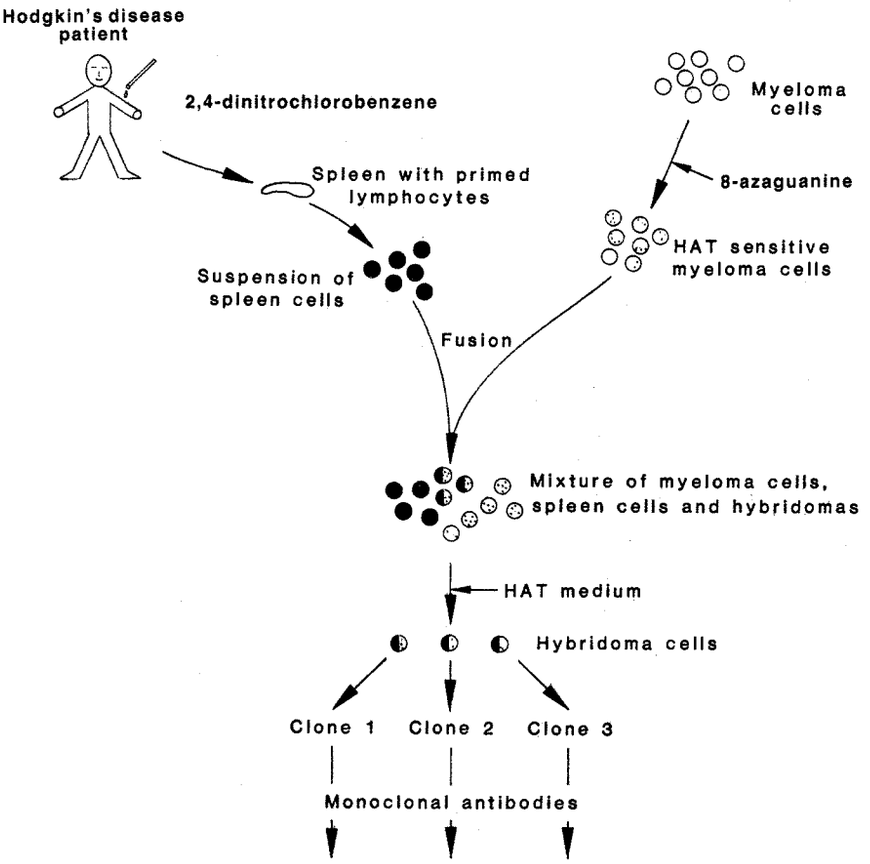
"We knew other workers had been plagued by mycoplasms, including Milstein and Köhler," says Kaplan, "so naturally we checked our cleaned-up cells before beginning our initial experiments." Those tests were negative. "The problem is," he explains, "that mycoplasmas grow very slowly, so a minimal undetectable contamination can later proliferate to become a major hindrance." This, apparently, is what happened with the Stanford cells.

Until recently mycoplasma contamination of cell cultures was virtually insurmountable, but in the past couple of years techniques have been developed for eradicating the parasites. "We have now treated some of our cells," says Kaplan, "and they look to be clear of contamination, but we can't be fully certain until clones have been grown for a while and their numbers expanded."

The people who received the original cells and those who are awaiting delivery have been notified of the problem and of the measures for circumventing it. Within the past week Kaplan has heard that one recipient of the cells has already succeeded in eliminating the mycoplasmas.

Production of that first hybridoma was, however, just the beginning. Many challenges remain, two of which are particularly important. First, because myeloma cells are a cancerous form of bone marrow cells, they too can produce antibodies or parts of antibody molecules. This need not be an insuperable problem, because the required antibody can often be separated from the myeloma "noise." But it is a nuisance. Second, in order to obtain human monoclonal antibodies of any required specificity, it will be necessary to develop new ways of collecting and priming human B lymphocytes to selected antigens. Kaplan describes success in this challenge as "the crucial step."

The question of dealing with antibody "noise" from myelomas has been solved with mouse cells. There are already several lines of mouse myeloma cells that produce no antibody at all and yet will readily fuse with mouse spleen cells to yield an antibody-producing hybridoma. "I'm hopeful that when we put our minds to it we will succeed in getting a complete nonproducing human myeloma," Kaplan says. So far the Stanford team has come up with virtual nonproducers, but there has always been a smidgen of antibody present, sometimes detectable only by the most sensitive instruments. But the cells are still producers.



Production of human monoclonal antibodies

Primed lymphocytes (a suspension of spleen cells) are fused with HAT-sensitive myeloma cells to give a mixture of hybridomas and unfused myeloma and spleen cells. The hybridomas are selected out, separated into wells, and grown. Their products are monoclonal antibodies.

"There are other ways to go on this one," Kaplan suggests, "starting, for instance, with a slightly different type of tumor cell, a lymphoma." Work along these lines looks promising, but it remains to be seen whether hybrids will be able to secrete any antibody that they might make. Kaplan is, however, confident that some kind of solution will be reached. "One way or another we will wind up with a nonproducer that we will be able to use some time during the next year."

The problem of devising effective techniques for priming antibody-producing cells in the laboratory has not been explored very thoroughly in mice, simply because it is less necessary than it is for human cells. Adding the caveat that he cannot yet say very much about the work with human cells, because it is still in preliminary stages, Kaplan notes that he has had some success with test tube systems. First he and Olsson primed human spleen cells by mixing them in a test tube with sheep red blood cells. They then moved on to lymphocytes that can be separated from normal peripheral blood. Success here too. "This is obviously a much more convenient system, because you can do it with just 20 milliliters of blood," Kaplan observes. "Once a system is perfected for effective sensitization of lymphocytes in vitro, everything else will fall into place."

Antibodies are supremely specific probes. An experimentalist who has access to a suite of monoclonal antibodies can therefore embark on molecular dissection of biological systems that was undreamed of until Milstein and Köhler's paper hit the scientific press in 1975. This potential is beginning to turn into reality, as too the dreams of clinicians who will want to attack infectious diseases and cancer in entirely novel ways. And monoclonal antibodies have edged close to the world of commerce with their application in extremely high purification of the antiviral protein interferon. The possibilities are legion.

But, warns Kaplan, there is a staggering amount of work ahead. "The Köhler-Milstein technique was a fantastic achievement of great importance. Yet, with the wisdom of hindsight you could call it a horse and buggy procedure: it was terribly complicated. A few years down the line from here one may look back and shake one's head, wondering how they ever succeeded because it was so complicated."—ROGER LEWIN