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当代免疫

Immunization Update

Where are we and where are we going in the battle against viral diseases immunodeficiency diseases and malignancies?

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编者按 美国乔治华盛顿大学医学中心 Ariel C. Hollinshead 教授是美国著明的病毒免疫学家，在国际上享有盛誉。他以免疫调节的观点在研究抗病毒病、抗免疫缺陷性病及抗恶性肿瘤方面，取得了较大的成果。由于她成绩卓著曾被评为美国科学家精英。在该文中，她对肝细胞癌、免疫缺陷性病，以及肺癌等疾病的免疫性机理作了精辟的论述，特别是对免疫调节方法治疗疑难疾患提出了新的展望，为了保持作者原意，促进国际交流，本文以原文发表。

Introduction

Successful vaccine programs have almost eradicated poliomyelitis from the Western Hemisphere. In the Eastern Hemisphere, mainly in Asia and Africa, polio still causes more than 200,000 cases of paralysis every year. Rare cases of paralytic polio also occur in recipients of the live-virus vaccine here in the West, and this form of vaccine is heat-sensitive and would be difficult to keep cool during transport in many parts of Africa and Asia. Thus, the Salk killed vaccine will probably be the choice of doctors as the World Health Organization gears up for a campaign to eradicate the disease by the end of the decade. The final triumph through worldwide use of the polio vaccines, first introduced in 1954, will have taken, therefore, more than forty years.

In 1962, workers succeeded in the first separation from membranes of animal tumor cells of polypeptides which, after meticulous screening, were identified as the antigens which induced cell-mediated immunity against virus and chemically-induced malignancies in animals. By 1965, these workers were

testing human tumors and identifying similar tumor antigens in man, Clinical trials with these membrane products of oncogenes have indicated that specific active immunotherapy with the Hollinshead TAAs (tumor-associated antigens) is safe, is effective and deserving of worldwide clinical trial evaluation

In part influenced by such pioneering efforts in poliomyelitis and in cancer, and in part due to emerging biotechnologies, there have emerged a wave of new attempts to eliminate certain diseases by immunization with a host of candidate agents. The wars on polio and cancer are similar to earlier success in smallpox eradication, because these vaccines are capable of inducing lifelong immunity. Many scientists miss this important point, puring the discovery, testing and evaluation of various candidate vaccines. Lifelong immunity is induced because 1)the vaccines are designed to be polyvalent; that is, they induce the body to form defenses against several antigenic variants and/or strains of virus, and 2)the vaccines are delivered in such a way as to induce strong, immediate but transient humoral immunity, coupled with steadily increasing cell-mediated immunity which peaks and plateaus after the last immunization, and lasts for a very long time in most of the vaccine recipients.

We will examine 1)the attempts to prevent a cancer associated with hepatitis B viral infection, namely hepatocellular carcinoma or primary liver cancer, and 2)the attempts to treat or prevent a fatal infection called acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV), and 3) the attempts to treat or prevent the most predominant and serious form of cancer, so far not associated with any virus, namely the several types of lung cancer. Our update review of topics 1) and 2) will be of work by others, and of topic 3) of work in our own laboratory; each topic will be followed by a few comments aimed at consolidating what has been learned by comparing the experiences in the three areas of study.

Hepatocellular Carcinoma

Although we do not understand the role of the virus, it is clear that hepatomas usually develop in patients with some form of chronic liver disease, and that in 80% of these patients there is a history of persistent hepatitis B viral infection (HBV). Primary hepatomas are more common in males, the frequency of occurence increases with age, the risk increases by 250-fold in hepatitis B-positive carriers, and is six times more prevalent in Asia and Africa. When the tumors are examined, at least seven partial copies of the HBV genome are present,but the sequences appear to have been altered or have conformational changes after integration. The cellular sequences all seem to have

in common the region seen at the 5′ end of the surface antigen gene. Just how the virus might cause the development of a cancer cell is not understood. It is probably not the development of a direct alteration of the cell's regulatory mechanisms, since there is such a long period after the initial infection. In the case of some retroviruses, oncogenes which were originally of cellular origin are expressed when the virus infects another cell. The small DNA papova- and adeno-viruses can integrate into the cell genome causing loss of growth control. It is also probably not like Burkitt's lymphoma, where the EBV infection causes mutagenesis through chromosomal translocations. This model is of interest because it involves cellular factors, but the evidence so far does not indicate this role for HBV. Another possibility might be a stepwise progression, where some cells may be partially transformed during early infection and be induced to progress at a later stage.

Several large-scale and many small-scale immunization programs are in progress using whole virus, parts of virus, subunits of virus, viral antigens, recombinant or chemically synthesized vaccines, hybrid particles and anti-idiotype antibody vaccines. An argument against the first three types of vaccine is that these preparations may contain contaminating antigenic components not relevant to the induction of immune responses. But, in purification processes, it is not known whether the last five types of vaccines listed (above) might have lost certain important or relevant components. Since HBV cannot be grown in cell culture, one easy alternative form of vaccine, the noninfectious surplus protein virus coat separated from plasma of asymptomatic carriers, HBsAg, as purified 22 nm spherical surface antigen particles, has been licensed and shown to be safe, free of AIDS or other agents in blood, and effective in doses of 20 meg.(micrograms) per ml. Recombinant yeast hepatitis B vaccines are also safe and effective, and antigenic in doses of 10 mcg/ml. Both micelles of native HBsAg from plasma and yeast-derived polypeptide micelles were more antigenic, and, more importantly, had an increased immunogenic potency. But, something even better has been developed, namely, subunit or polypeptide vaccines as micelles.

The immunogenic polypeptides, p25 and p30 have been removed by a special detergent process from the viral coat and aggregated so that the hydrophobic regions are sequestered in the interior of the particles with the hydrophilic residues on the surface, so that the resulting forms are water soluble (protein micelles). Both safety and protective efficacy studies have been completed in primates and clinical trials are in progress. More recently, polypeptide micelles have also been prepared from these same viral surface antigens

expressed by recombinant yeast and mammalian cells.

In the construction of hybrid vaccinia vaccine, the foreign viral DNA is introduced into the vaccinia DNA by first making chimeric genes consisting of vaccinia virus promoter sequences ligated to the coding sequence for the desired foreign protein which are then flanked by vaccinia DNA in a plasmid vector. In chimpanzees, the vaccine administered by multiple pressure or the scratch technique induced a secondary antibody response when challenged intravenously with live heterologous subtype HBV, showing that they were immunologically primed, although the vaccine itself induced no circulating surface antibody. Since at present there are no accepted laboratory markers of attenuation or of virulence of vaccinia virus for man, it is not known whether changes in host range or tissue tropism of vaccinia viruses may occur and whether alteration of virulence can be predicted.

Synthetic vaccines were first demonstrated with tobacco mosaic virus, when the antigenic determinant responsible for the immunogenic activity of the virus was identified, sequenced, and the amino acids were coupled to a carrier protein, inducing neutralizing antibody in experimental animals. A dominant epitope in positions 141—146 of HBsAg was analyzed and synthesized, and this synthetic peptide was attached to aldehyde-stabilized human erythrocytes and injected into mice. It induced the formation of hepatitis B surface antibody with and without the use of Freund's complete adjuvant (FCA). In another study, two hydrophilic regions of HBsAg, consisting of two cyclic peptides containing disulfide bonds in the region 117—137 and 112—137 were incorporated into several adjuvants, including FCA, alum, and multilamellar liposomes with and without muramyl dipeptide. Groups of BALB/c mice were immunized intraperitoneally. Hepatitis B surface antibody was induced 7 to 14 days after inoculation in approximately 50% of mice in each group, and, in 75 to 80% of mice when the 117—137 peptide was emulsified with Freund's complete adjuvant. Since this was a single injection without carrier protein, the peak levels of antibody declined after day 21 in most mice. No attempt to measure cell-mediated immune responses was made. Other experiments with other sequences were performed, and 7 of 13 both free or protein carrier-linked synthetic peptides elicited antipeptide responses in rabbits, with the best carrier protein being keyhole limpet hemocyanin in FCA and subsequently in IFA. Another report was of interest; a peptide with sequence 110—137 stimulated a transitory antibody response to they surface antigen determinant, in chimpanzees, but repeated immunization failed to induce stable surface antibody response. Nevertheless, intravenous challenge with HBV with the ayw determinants

late in the immunization course resulted in protection against infection in one of three chimps. Recently, a protein sequence which mediates the attachment of HBV to human hepatoma cells was identified. A synthetic peptide analog, which is recognized by both cell receptors and viral antibodies, elicted antibodies reacting with the virus. Such a peptide might block attachment of virus to cell.

The question is raised as to whether or not mixtures of more than one of the peptides may be required. A peptide spanning the pre-S2 region was synthesized and coupled to keyhole limpet hemocyanin and emulsified in IFA supplemented with sodium selenite. This vaccine protected two chimpanzees against challenge with 10 to the sixth power infectious doses of HBV. The same peptide bound to spherical 22 nm surface antigen particles is now being tested using FCA, since protective immunity which persists is best obtained using FCA.

The expression and secretion of hybrid envelope particles by established cell lines may provide an efficient system for the production of potential new vaccines. Insertions of envelope proteins of HBV carrying the surface antigen and in-phase inserted polio type 1 sequences were assembled with cellular lipids in cultured mammalian cells after transfection. The inserted polio neutralization peptide was found to be exposed on the surface of the hybrid envelope particles and induced neutralizing antibodies against poliovirus in mice immunized experimentally. Another potentially excellent carrier is the core particles of HBV. The core structures induce antibody and augment T-helper cell functions.

Finally, anti-idiotype antibody vaccines (the idiotype binds to the epitope part of the antigen) have been used together with surface antigen or synthetic HBV peptides to enhance the immune response to the surface antigen. Also, patients repeatedly immunized with human anti-HBs antibody induced anti-idiotype antibodies.

Comments

In our preliminary studies of hepatomas, we identified individual tumor cell membrane proteins which produced excellent cell-mediated immune responses. These studies involved a screening program to test CMI capabilities of candidate polypeptides soluble and separable from interfering or blocking substances on tumor cell membranes. Perhaps, for example, these tumor-associated antigens (TAA) should be coupled with 110—137 or with HBV attachment protein, or with both. With properly chosen adjuvant, and with

properly chosen route of immunization, such combination immunoprophylactic or immunotherapeutic biologics might form a more perfect candidate immunogen to test for protection of carriers against further infectious side-effects, liver damage and subsequent malignancy. Since this is one of the top ten cancers in the world, the suggestion is not an idle one.

Acquired Immunodeficiency Syndrome

Human immunodeficieny virus (HIV) belongs to the family Retroviridae, subfamily Lentivirinae. Lentiviruses are named because of their typically slow progression from infection to over disease. HIV was independently discovered by researchers in France in 1983 and in the USA in 1984. Originally called lymphadenopathy-associated virus or human T-lymphotropic virus type III, or AIDS-associated retrovirus, it was finally named HIV, and a related, but genetically distinct virus, HIV-2, was identified as the cause of AIDS, as distinguished from HIV-1 virus. The Center for Disease Control developed a case definition for AIDS, revised in 1987. Persons with AIDS, whose immune systems are compromised, have opportunistic diseases. Examples that are life-threatening for persons with AIDS but not for normal persons are Kaposi's sarcoma and Pneumocystis carinii pneumonia. HIV-infected persons who have no opportunistic infections or diseases have symptomatic HIV infection. When serologic tests show antibodies to HIV, but no signs or symptoms, the infected person is said to have asymptomatic HIV. In June 1988, the CDC recommended universal precautions to health care personnel which consider that blood and certain body fluids are potentially infectious, and are intended to help prevent parenteral, mucous membrane and nonintact skin exposures to these fluids. In summary, HIV infection is a progressive disease process associated with a spectrum of illness. We are fighting an epidemic of HIV infection rather than AIDS, which is the endstage of this progressive illness.

HIV is a genetically variable virus, and neutralizing antibodies raised against one HIV-1 isolate do not cross-neutralize with other HIV-1 isolates. The virus is capable of spreading from cell to cell without entering the fluids and can escape neutralization. It can remain latent in the body for long periods, probably as an integrated provirus in the genome of the host cell. HIV envelope glycoprotein appears to be poorly immunogenic for primates and elicits weak antiviral antibody titers, perhaps due to epitopic suppression or to cell blocking or interfering factors. Although we speak of the two serotypes, HIV-1 and -2, each virus type consists of many variants, with up to 25% or more sequence divergence among the variants.

The major neutralization epitope of HIV-1 corresponds to one of the hypervariable regions of the env glycoprotein. If an HIV vaccine is to be efficacious, a great number of different neutralizing antibody species will have to be elicited. Although, no one has tested it, the same criteria may possibly apply to the cell-mediated cytotoxic immune response. The way in which the virus escapes the host immune response, such as cell-to-cell spread and latency, means we may have to cope with the fact that the virus may remain in tissue as an integrated provirus, or perhaps as intact particles inside cytoplasmic vacuoles in macrophages. The virus has been found sequestered in the central nervous system and in bone marrow cells. Some immune responses that are naturally triggered by the virus in seropositive patients may have an adverse effect on protection against infection, and such substances may be inadvertently triggered as a consequence of vaccination. HIV produces a protein called Tat, which promotes the growth of cells cultured from Kaposi's lesions. This is not the first instance of a viral protein which is associated with tumor cell proliferation, but further study may shed light upon the way in which it may affect tumor growth. HIV multiplies in T4 lymphocytes that are activated through contact with specific antigens. Targets of viral antigen therapy might be the very T4 cells educated to respond to HIV; this could lead to strong inhibition of the immune response which should have helped eliminate viral infection. Cells replicated HIV shed gp120 molecules that can bind the CD4 recentirs on uninfected T4 lymphocytes. It is not known whether a vaccine inducing an immune response against env antigen alone would be objective, but this is under clinical testing. Some workers think there may be advantages to adding other antigens such as are gag, pol, or some of the nonstructural HIV proteins.

Recombinant vacinia viruses expressing gp160env or p25gag, another preparation containing formalin and betapropiolactoneinactivated HIV, and a purified gp 160evn and p18gag antigens in a MDP-base adjuvant formulation have all been tested in chimpanzees. No neutralizing antibodies were detected after immunization with the live recombinant virus vaccine. An IV booster with autologous T cells infected in vitro with recombinant vaccine induced no neutrlizing antibodies. A killed HIV preparation in an MDP-base adjuvant was injected and the antibody titers rose after this booster injection at 7 months. The best chimp responder had a 1 : 50,000 antibody titer to gp160, p25 and p18gag by Western blot and a 1 : 5000 titer to gp120. The Elisa antibody titer reached 1 : 102,400 within two weeks after the boost. In parallel, strong indirect ADCC activity was detected, and the chimp maintained its T-cell pro-

liferative response. The chimp was challenged by IV injection of 32 $TCID_{50}$ of a LAV-1 virus stock and did not resist the challenge. Results from ongoing human trials suggest that gp120 is a poor immunogen and that HIV neutralization epitopes are poorly recognized by the primate immune system. A mixture of a titated HIV-1 isolate and serum from an infected to establish infection in a naive animal, but whether this is due to the protective effect of neutralizing antibody or to other serum components is not clear. Uninfected mangabeys died within 2 weeks of injection with a lethal SIV variant, whereas naturally SIV-infected mangabeys remained alive and healthy. This means it is possible to protect at last against disease with a hypervirulent SIV by previous infection with a related virus isolate. A killed prototype SIV vaccine conferred partial protection to rhesus macaques (two of six) against a SIV virus challenge.

Comments

Some of the pieces of the HIV vaccine puzzle are in place. Nowhere are the provirus proteins mentioned in detail. It may be very important to study the early non-assembly proteins induced by HIV. Also, as indicated, the strongest viral or viral antigen substances need to be studied in association with early non-assembly proteins or with certain early infected cell membrane proteins to determine the effect of these combinations in inducing long-lasting cell-mediated immunity. In addition we cannot ignore the importance of proper adjuvants and effective routes of administration, as well as the proper timing of the immunogen combinations. In the eventual larger trial, subjects must be selected as a group most likely to respond. The results of such a large clinical study will inevitably give the further information needed for advanced stage treatments.

Lung Cancer

Summarized below are I. the immunogen TAA (tumor-associated antigens) used for lung cancer immunotherapy, II. the methods used in the administration of these immunogens in clinical trials of specific active TAA immunotherapy, III. the results of clinical trials of specific active immunotherapy for lung cancer, IV immune response monitoring evaluations and what they indicate, and V. the way in which certain drugs, selected for their action in the immune system, may be synergistic with specific active TAA immunotherapy, combination therapy, especially for resected patients of later stages.

I. TAA description: Five major types of lung cancer TAA biologic drugs-

immunogens were prepared. Tests in patients to compare single and combination TAAs permitted the selection of the strongest synergistic effect per given protein concentration. The TAA immunogens were prepared in batches with good reproducibility between batches, and with careful quality control. The contents for each lung cancer type were:

TAA Immunogens	Molec. Weight	Description
Oat cell	51kDa	unique* protein
	69kDa	unique lipoprotein
Large cell	37kDa	unique protein
	51kDa	unique protein
Squamous cell (epidermoid)	37kDa	unique protein
	49kDa	fetal protein
	100kDa	fetal lipoprtein
Adenocarcinoma	51kDa	unique protein
	77kDa	unique protein
Bronchioalveolar	77kDa	unique protein
	100kDa	fetal lipoprotein

* (not found in this form in normal lung)

Note that some antigens are shared between these strictly evaluated histologic types. Yesner and Hollinshead have reported the substantiation of the "Y-theory" of the origination of lung cancers, since the patterns of the above TAA biomarkers coincide remarkably with the postulation that all lung cancers have a common origin in the endoderm. As supported by other evidence, the Y concept has small cell carcinoma at the base of Y, large cell carcinoma at the fork, and at each arm are squamous cell carcinoma and adenocarcinoma. Note the pattern of shared antigens amongst the TAAs as consistent with this theory. Synergistic TAAs of bronchioalveolar carcinomas include 77 kDa marking this subclass of adenocarcinoma, and 100kDa as shared with squamous cell carcinomas, perhaps related to the strong keratin expression of pneumonocytes.

II. Administration of Immunotherapy:

TAA immunogens are well-homogenized with adjuvant, and administered intradermally once per month for three months. The first immunization is given about 2 weeks after surgical removal of lung tumors. The principle of active specific immunotherapy is based upon the well-described, classic dual effect of appropriate immunization. Proper emulsification of antigen and adjuvant is crucial. We have used 500 mcg TAA immunogen in 0.2 ml emulsified with

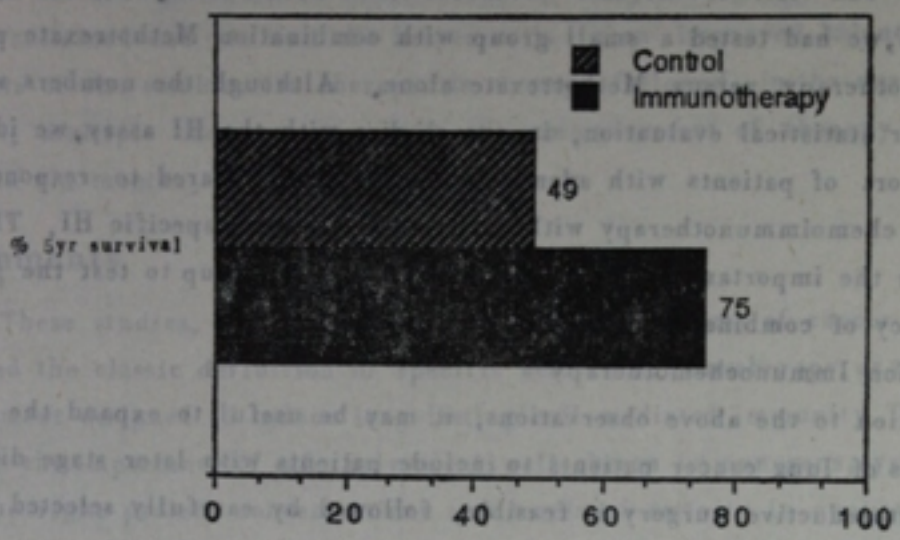
an equal volume of complete Freund's adjuvant, but have learned that a good emulsification can be obtained using 0.1 ml adjuvant, and that 300 mcg TAA per 0.2 ml is adequate. We usually administer the emulsified immunogen in three adjacent sites, a triangle formation, on one arm the lst month, another arm the 2nd month and on the thigh the 3rd month. Physicians are warned that any usage of steroids may interfere with the efficacy of this therapy. The first action induced by immunization is an immediate release of the aqueous portion of emulsion, followed by boosting from the emulsified portion. Classic studies show that a well-prepared immunogen resides at the site of injection for several months, is constantly transported to subpleural parts of lungs and is detected not only in regional but also distant lymph nodes. So far, other adjuvants have not been shown to induce long-lasting cell-mediated immunity, the Keystone of classic active specific immunotherapy.

Responders to immunotherapy mount specific immune responses which last even as long as 12-14 years. Toxicity includes small skin ulcers at the sites of immunization, similar to that seen for smallpox vaccinations, and these generally heal within 4 to 6 months; a possible overnight fever may occur, although emulsification reduces the tendency toward febrile reactions. There are no long-term toxic effects, specically no autoimmune reactions, using these highly purified ponlypeptides, essentially free from nucleic acids, major tissue antigens, pyrogens, bacteria and viruses.

III. Clinical Studies.

After a successful Phase II clinical in stage 1 lung cancer patients, two Phase III clinical trials in stages 1 and 2 disease were conducted. Each of these trials have been reported separately, and are described in detail in well-referenced overall reports. Except for the inclusion of stage 2 disease in the Phase III trial, the common features of the three clinical trials were the same. The selection criteria included: no previous treatment prior to curative resection; no history of other malignancy in the past five years; contraindications were pregnancy or stages 3 and 4 disease; stage 1 and 2 disease fully resected with all excised lymph nodes diagrammed and numbered. Staging criteria included surgical and pathology reports, American Joint Committee TNM staging, as well as a final overview of pathology with review of records and slides and final staging determination. The eligible patients were strictly randomized into control and therapy groups, and the follow-up to be after five years. The actual five-year survival is show below:

Lung Cancer Specific Acive Immunotherapy-stages 1 and 2



234 Patients, 116 Controls, 118 Immunotherapy
(1/88 data)

Distribution according to stage, including two patients with T_2N_2 initially staged incorrectly, were as follows;

	Controls	Immunotherapy
T_2N_2	0	2
T_2N_1	17	22
T_1N_1	9	13
T_2N_0	43	44
T_1N_0	47	37

P values as assessed by both Wilcoxon and Savage tests were all below 0.00. Kaplan-Weier density analyses were highly significant. Optional studies in individual trials included an analysis of a group using adjuvant alone, and ne statistical difference from the control group was seen at 5 years.

IV. Immune changes

We have reported in detail measurements of cell-mediated immunity and measurements of humoral immunity by controlled, standardized tests. There was a striking correlation between patients with long-lasting CMI and stong early humoral responses to immunotherapy. The latter test of HI employed a reverse enzyme immunoassay utilizing serial sera from patients before, during and after immunotherapy versus purified, separated, well-characterized TAA epitopes. This assay appears to discriminate between patients who respond to therapy and those who fail therapy 5-6 months after commencement of immunotherapy. This will permit us to try other therapies in such patients, with options such as a combination immunochemotherapy, booster TAA shots, additional polyva-

lent vaccins, and other strategies, since there is still time to try different approaches. The tests of HI also revealed other useful data. In our first clinical trial, we had tested a small group with combination Methotrexate plus TAA immunotherapy versus Methotrexate alone. Although the numbers were too small for statistical evaluation, in our studies with the HI assay, we identified a cohort of patients with adenocarcinomas who appeared to respond to combination chemoimmunotherapy with earlier and stronger specific HI. These data indicate the importance of a larger trial in this subgroup to test the possible efficacy of combination therapy.

V. Combination Immunochemotherapy

In addition to the above observations, it may be useful to expand the clinical studies of lung cancer patients to include patients with later stage disease where cytoreductive surgery is feasible, followed by carefully selected immunochemotherapy. Neither chemotherapy or immunotherapy of solid tumors has been shown to have a predictable effect upon B and T cells or patients in patients with solid tumors, especially when measured within the first half year of therapy. However, we have observed that the combination of both therapies, using drug and dosage carefully chosen for effect upon the immune system, may have a dramatic effect in resected later stage patients. An example is shown below,

Helper/Suppessor Cell Changes

Adenocarcinoma of Lung

60F, multiple metastases, ICTx

	CD4/CD8	CD4	CD8
6/13 PreTx	2.71	8.19	3.02
8/18-19 (Mtx + Citrovorum Rescue)			
8/22	0.21	7.0	33.0
8/23 (300mcg TAA + FCA = 0.3ml)			
9/12	3.24	8.47	2.61
9/19 (TAA + FCA)			
10/10 (22days post Mtx)			
	6.36	9.34	1.46
10/17 (TAA + FCA)			
12/14 multiple metas. disappeared			

Note that the effect of Methotrexate, as we have reported previously, is to cause a rebound overshoot, to flush-up the white cells. Of great interest is the fact that in this patient there appeared to be a large component of occult suppressor cells which made up the bulk of the cell overshoot response. The

use of TAA specific active immunotherapy, when given at the proper time, at the overshoot, 'educated'n the flused-up cells, and in combination with the drug sustained and restored the helper cells to an improved balance. Clinical effects of the combination therapeutic strategy followed, in the example shown above, multiple metastases present at commencement of therapy disappeared within six months.

Comments

These studies, and similar studies with other forms of cancer, have followed the classic definition of specific active immunotherapy as that form of treatment designed to induce long-lasting cell-mediated immunity. These studies stress the importance of safe, polyvalent, well-chosen immunogens, synergistic and at the right protein concentrations and levels of activity in quality-controlled batches for clinical use. As the leading cause of cancer in the Western Hemisphere, and as a major form of cancer in the Eastern Hemisphere, these gains in survival benefit are a real treatment advance, and statistically evaluable, especially the phase II Canada and phase III USA trials, as worthy of further large scale evaluation and usage for patients with lung cancer. However, the preparation of large batches of TAA immunoges is expensive, and awaits commercial enterprise.