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The design of customized and the modification of existing standard robot hardware have been completed and the automated system is now capable of carrying out the tasks necessary to complete the amino acid analyses and protein primary sequence analyses (including protein hydrolysis and derivatization). The robotic amino acid analysis system has now been validated and shown to be capable of analyzing proteins in the 17kD range. The proposed modification of these procedures for endotoxin detection have been shown to be appropriate. These studies have demonstrated that once the complex series of steps involved in carrying out a chemical analytical procedure are reduced to a series of modular operations, these procedures can be performed by a robot. Once these modular operations have been automated they can be used in any configuration to carry out other defined tasks consisting of the same modular operations and with the appropriate hardware the robot can carry these procedures out on a micro scale.

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FINAL REPORT

GRANT #: N00014-90-C-0046 R&T CODE: 213f002

PRINCIPAL INVESTIGATOR: J. Wayne Cowens, Co-investigator: M. Jane Ehrke

INSTITUTION: Health Research Inc., Roswell Park Division

GRANT TITLE: Application of Laboratory Robotics to the Determination of the Primary Structure of Recombinant Proteins and the Measurement of Endotoxin

AWARD PERIOD: 1 NOVEMBER 1989 - 31 October 1992 Extended without further funds until 30 September 1994.

OBJECTIVE: (1) To develop a robotic system that can be used to automate the procedures used to determine the primary structure of a recombinant protein and the presence of endotoxin and (2) to apply this system to the development of quality control procedures for polypeptides manufactured as therapeutic agents by the pharmaceutical industry and to the study of the primary structure of the Rh(D) epitope.

APPROACH: (1) The procedures used to determine the primary structure of a protein consists of a series of discrete chemical reactions for each analysis involving multiple modular operations (e.g. pipetting, weighing, mixing, heating, evaporating, purging). Since a robotic system is capable of performing a series of modular operations in any order, the automation of these procedures was undertaken. Starting with a Zymark System V controller and a Zymate II+ robot arm, standard Zymark robotic stations were modified to accommodate the chemical reaction chamber-tube assembly, and custom stations for controlled heating, vacuum evaporation and purging with inert gases were developed in collaboration with Zymark. The new software required for the robot to interact with the modified and customized Zymate system were written. (2) Using the system developed as described above all the chemical reactions involved in amino acid analysis and preparation of peptide samples for primary sequence analysis by GC-MS were adapted to the robotic system. Once all the individual steps were successfully carried out robotically the automated analyses were validated.

ACCOMPLISHMENTS: The fabrication and testing of the custom hardware modules in collaboration with Zymark was completed. The low level software that allows the robot to make use of the custom modules to carry out the desired function of each was completed. The high level software that allows the robot to carry out the individual steps in the derivatization procedure for amino acid analysis was written. The robot was interfaced with an HPLC system. Complete amino acid analysis of a single sample of a 10-mer peptide, rH-TNF residues 34-43, was successfully accomplished robotically from drying the original sample to injection of derivatized

hydrolysate into the HPLC. Problems encountered when multiple sample analysis was undertaken were solved by software rewrites, modification of the custom vacuum station and adaptation of a custom heating station. The robotic system for multiple amino acid analyses was validated using the 10-mer. The limit of detection has been determined to be 0.16µg. Reproducible, accurate results have been obtained with 0.8-10.0µg of starting material. The derivatization chemistry for peptide sequencing was modified in order to facilitate automation. The high level software that allows the robot to carry out the individual steps in the derivatization procedure for peptide sequencing was written. The robotic system was tested using a 19-mer, rH-TNF residues 110-128, and rM-TNF. Problems were identified that required further modification of the custom vacuum station and the replacement of solvent manipulating instruments with ones made out of more inert materials. The robotic system has now been used to successfully carry out partial acid hydrolysis and derivatization chemistries on eight peptides ranging in size from a 6-mer to a 21-mer. The derivatized hydrolysates were analyzed by the automated GC-MS-DS system. Information defining 70 to 100% of the primary sequence of each was obtained. During this period the derivatization procedure for peptide sequencing was successfully modified to detect endotoxin. Starting with 0.8µg of LPS (*E. coli* 0111:B4, Difco) it was possible to detect $\geq$ 15ng of endotoxin. Finally the transfer of this prototype equipment/technology to the US Food and Drug Administration for the purpose of evaluating its potential benefit has been accomplished.

CONCLUSIONS: The design of customized and the modification of existing standard robot hardware have been completed and the system is now capable of carrying out the tasks necessary to complete the amino acid analyses and protein primary sequence analyses (including protein hydrolysis and derivatization). The robotic amino acid analysis system has now been validated and shown to be capable of analyzing proteins in the 17kD range. The proposed modification of these procedures for endotoxin detection have been shown to be appropriate.

SIGNIFICANCE: These studies have demonstrated that once the complex series of steps involved in carrying out a chemical analytical procedure are reduced to a series of modular operations, these procedures can be performed by a robot. Once these modular operations have been automated they can be used in any configuration to carry out other defined tasks consisting of the same modular operations and with the appropriate hardware the robot can carry these procedures out on a micro scale.

PATENT INFORMATION: A patent application on the custom vacuum/purging station and copy right applications on all the software are being considered.

PUBLICATIONS AND ABSTRACTS (for total period of grant):

1. Pocchiari, S., Mead, L., Reino, M., Ehrke, M.J., and J.W. Cowens. (1990) Detection of endotoxin using an automated gas chromatography-mass spectrometer (GC-MS) system. Abstract. Fourth Symposium of the Protein Society. T105.

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5. Reino, M., Diegelman, P., Pocchiari, S., Ehrke, M.J. and Cowens, J.W. (1991) Development of a system to automate primary structure determination of recombinant proteins utilizing a custom Zymate II. Proc. Intl. Symposium on Laboratory Automation and Robotics, pg. 63.

6. Pocchiari, S., Ehrke, M.J., Mihich, E., and Zaleskis, G. (1993) Differential effects of cyclophosphamide (CY) alone and in combination with tumor necrosis factor (TNF) on murine thymocytes. J. Immunol. 150: 109A.

7. Ehrke, M.J., Pocchiari, S., Wollman, R., Cowens, J. W., Mihich, E. and Alderfer, J. (1994) Structure/activity studies identify a specific 3-dimensional domain linked to TNF-mediated thymocyte apoptosis. Cytokine 6: 566.

CYTOKINE, Vol. 6, No. 5 (September 1994: 539-582)

A162

A POSSIBLE ROLE OF A THYMOCYTE GROWTH FACTOR IN POSTRADIATION RESTORATION OF THYMUS.
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Inst. of Immunology, Moscow, Russia 115478
The thymocyte growth factor (THGF) is a secretory product of mouse cell line TC.SC-1/2.0 which has the phenotype of intrathymic SC-1$^+$ PNA$^+$ L3T4$^-$ Lyt-2$^-$ T-lymphocyte precursors (TLP). It was shown THGF is secreted by cells of this line spontaneously and radiation at doses 10-12 Gy increased production of THGF to a marked degree. On the other hand thymocytes of CBA mice which were accumulated in thymus 2-5 days after total-body radiation at a sublethal dose (probably SC-1$^+$ PTL) also produced spontaneously THGF - like activity and responded by increase of proliferation to THGF and IL-3, and thymocytes which were radiated in vitro at doses 10-50 Gy responded to THGF but not IL-2. Injection of THGF to these mice stimulated simultaneously the differentiation of THGF - and IL-3 - dependent PTL and migration of the mature cells from thymus. Thus, in vivo THGF production is activated probably by damaging factors in particular radiation, and target cells for THGF are radioresistant cells of thymus which serve by a source of early restoration of this organ.

A163

CLONING AND CHARACTERIZATION OF A NOVEL CHEMOKINE--LIKE CYTOKINE.
A. Zlotnik, J. Kennedy, K. Bacon, S. Kleyensteuber, T. Schall, and G.S. Kelner
DNAX Research Institute, Palo Alto, CA 94304
A novel cytokine was cloned from a mouse PRO-T cell cDNA library. The nucleotide sequence of this molecule, designated Lymphotactin, exhibits a significant degree of homology at the third exon with members of the C-C chemokine family. At the amino acid level, Lymphotactin has only two (C_2 and C_4) of the four cysteines characteristic of the C-C chemokine family. The biological activities of Lymphotactin detected so far include growth factor for a cell line (NFS-60) and potent chemotactic activity on some populations of B and T lymphocytes (but not on macrophages or neutrophils). These observations strongly suggest that this cytokine represents a new class of chemokine.

P7 October 4: Apoptosis

A164

STRUCTURE/ACTIVITY STUDIES IDENTIFY A SPECIFIC 3-DIMENSIONAL DOMAIN LINKED TO TNF-MEDIATED THYMOCYTE APOPTOSIS. M.J. Ehrke, S. Pocchiari, R. Wolmann, J.W. Cowens, E. Mihich, G. Zaleskis, and J. Alderfer; Depts. of Exptl. Therapeutics and Biophysics, Roswell Park Cancer Institute, Buffalo, NY 14263
This study examined the influence of possible structure/activity relationships in determining the multifunctional nature of cytokines, using tumor necrosis factor (TNF) as the model. Specifically, could different domains along the linear sequence elicit different biological responses, and did a synthetic polypeptide spanning that linear sequence retain the three-dimensional (3-D) structure of the domain in the intact protein. A synthetic peptide spanning the 21-amino-acid sequence of human TNF from position 45 through 65 was unique among those tested in mimicking TNF-mediated induction of thymocyte apoptosis. The peptide did not induce other TNF functions nor did peptides spanning other sequences induce this function. The NMR and simulated annealing technique determined 3-D structure of this peptide indicated that it did not retain the structure it has within the intact protein. There are; however, some commonalities the residues 51-56 still form a loop-like structure and Glu-53 points directly out from the loop. It is postulated that this structure may be critical to the biological function. (Grant N-00014-90-C-0046, ONR, CA 13038 NCI and NY State funds)

A166

Apoptosis is not requried for IL-1ß secretion in stimulated human blood monocytes. Matthew J. Kostura*, Jayne Chin, Douglas Kawka Sol Scott and Irwin I. Singer Departments of Pharmacology and Biochemical and Molecular Pathology, Merck Research Laboratories, Rahway, NJ 07065

Because exposure of human monocytes (hMO) to lipopolysaccharide (LPS) suppresses apoptosis (D. Mangan & S. Wahl, J. Immunol. 147:3408-3412, 1991), apoptosis may not be obligatory for LPS driven IL-1ß secretion. To further explore the role of programmed cell-death in LPS mediated IL-1ß release, we determined whether IL-1ß synthesis and secretion are correlated with expression of markers of apoptosis in LPS or heat-killed *S. aureus* (HKSA) stimulated hMO. Left untreated, hMO begin to exhibit morphological and biochemical signs of apoptosis (plasma membrane blebbing detected with SEM, chromatin condensation seen with TEM, and endonucleosomal DNA breakdown observed by electrophoresis). Treatment of hMO with 50 pg/mL LPS (priming) suppresses the expression of these markers and stimulates synthesis, but not secretion, of IL-1ß. Treatment of primed hMO with increasing concentrations of LPS results in a dose-dependent release of IL-1ß, but does not increase DNA breakdown or membrane blebbing. Release of lactate dehydrogenase increases in an LPS dose-dependent fashion, but the percent of total LDH released is low (<2%) relative to levels of IL-1ß released (>40%) at 100 ng/mL LPS. Addition of cycloheximide, which induces apoptosis, increases the *rate* of LPS induced IL-1ß secretion by approximately 2-fold, but the total amount of IL-1ß released remains unchanged. In addition, IL-1ß is localized on intact cell-surface membranes of secreting (but not primed) hMO by immunoEM. These data suggest that LPS or HKSA mediated IL-1ß secretion is not associated with increased apoptosis, and that IL-1ß secretion occurs independently of cell membrane lysis in hMO.

A165

IFN-β , ds-RNA AND TNF INDUCE DNA FRAGMENTATION IN TARGET CELLS.
M.G.Gevorkian. The Institute of Molecular Biology, Academy Science of Armenia, Yerevan.

The earliest detectable event in cytosuicidal effect of simultaneous action of interferon-β and poly(rI)poly(rC) on human lymphoid leukemia cells and tumor necrosis factor on subcutaneous murine tumour cells are the increase of c_{AMP}, sustained increase in cytosolic free Ca^{2+} influx, activation of phospholipase A2 end release of lisolecitines and unsaturated fatty acids. These biocemical changes are followed by induction of Ca/Mg dependent endonucleolytic activity, that cleaves genomic DNA at internucleosomal sites, changes in poly ADP-rybosilation of histone and non-histon proteins, depletion of NAD^+ and ATP and chromatin condensation. The activation of Ca/Mg endonuclease is observed as a result of Ca^{2+} iones translocation into lymphoblasts nuclei by means of calmodulin.

A167

INCORPORATION OF ^{3}H-THYMIDINE (^{3}H-T) BY PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) FROM NORMAL SUBJECTS AND ACUTE MYELOGENOUS LEUKEMIA (AML) PATIENTS IS INDEPENDENT OF INTERLEUKIN (IL)-1β CONVERTING ENZYME (ICE) BLOCKADE
N. H. Margolis and Charles A. Dinarello, New England Medical Center Hospitals and Tufts University School of Medicine, Boston MA 02111.
The role of ICE analogues in programmed cell death led us to ask if this process is mediated by IL-1β. We used an analogue of IL-1β as an inhibitor of ICE (ICEi). The ability of ICEi to block the cleavage of precursor IL-1β was demonstrated by diminished levels of secreated mature IL-1β in LPS-stimulated normal whole blood samples (60% reduction) or PBMC cultures (68% reduction) which were pretreated with ICEi. PBMC stimulated with phytohemagglutinin showed no significant change in ^{3}H-T incorportation when pretreated with ICEi. LPS-stimulated whole blood samples and PBMC cultures from AML patients showed similar reductions in IL-1β, 77% and 65% repectively. LPS-stimulated PBMC cultures from AML patients showed no significant change in ^{3}H-T incorporation when pretreated with ICEi.
Given that ^{3}H-T incorporation indicates cell proliferation, these data suggest that the ability of PBMC to proliferate is independent of their ability to process IL-1β.